Morphological and molecular studies of diversity in clubroot caused by *Plasmodiophora brassicae*

Abdelwahab Mostafa Badi

M. Sci.

Garyounis University- Libya

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Declaration

In compliance with the university regulations, I declare that:

I. except where due acknowledgement has been made; the work is that of the author alone;

II. the work has not been submitted previously, in whole or in part, to qualify for any other academic award;

III. the content of the thesis is the result of work that has been carried out since the official commencement date of the approved research program;

IV. ethics procedures and guidelines have been followed.

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TABLE OF CONTENTS

Chapter 1. General Introduction and review of the literature

Chapter 7. Overview

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

Summary

P*lasmodiophora brassicae* Woronin is an obligate biotrophic plant pathogen that causes the disease clubroot in vegetable and oilseed crops in the *Brassicaceae* (cabbage family), particularly in *Brassica* species. Developmental changes in susceptible host plant cells lead to the development of root galls and stunted host plant growth. Clubroot is one of the most devastating diseases of vegetable brassicas worldwide and significantly reduces crop yield. In Australia, many attempts have been made to reduce clubroot spread, and clubroot is partially managed by using a combination of integrated control methods and a few resistant varieties.

Knowledge of the diversity of *P. brassicae* in Australia is confined to pathotyping tests on host plants. Australian populations of the pathogen have high pathotype diversity compared with other countries and these are not stable. This may be because of high underlying genotype diversity and instability, which can be tested by molecular methods. Breeding resistant cultivars is difficult if the pathogen is very diverse. It is also essential to understand the host-pathogen interaction by comparing the process of infection in susceptible plants (that develop large galls) with plants with some resistance (that develop no or late, reduced galls), so that critical stages in the progression of the disease can be characterized and potential targets for plant breeding of resistant varieties identified. Only *Brassica oleracea* (cauliflower) and *Arabidopsis thaliana* have been investigated microscopically after infection by Australian populations and features are not necessarily the same as those with populations from other countries and species.

In Chapter 2, variation in the pathogenicity of ten field populations of *P. brassicae* was studied using the European clubroot differential set (ECD) of *Brassica* hosts. These ten populations from two important crucifer production regions in Australia (Victoria and Western Australia) generated ten triplet codes; nine of these were similar to those found for previous Australian field populations but one was new to Australia. This high diversity was similar to that in previous Australian studies but was much greater than that reported in other countries. Populations showed no clustering with geographic origin or collection date using multivariate analysis.

Chapter 3 investigated the genotypes of these ten populations by the polymerase chain reaction (PCR) using six microsatellite and 60 randomly amplified polymorphic DNA (RAPD) primers. Reproducible amplification of DNA occurred with all primers used.

Pathotypes showed much genotype polymorphism with almost all primers tested and a smaller set of primers showing the most diversity was chosen. The high genotype diversity was similar to that found in some studies but not others in countries, fields and galls. Populations clustered with no apparent relationship to geographic origin, collection date or pathotype. One profile of a highly pathogenic population was distinctive but no marker for high or low virulence was found.

In Chapter 4, the stability of *P. brassicae* genotypes with different levels of virulence was investigated through three generations of plant passage by repeatedly inoculating and recovering *P. brassicae*. PCR profiles varied with each generation with all selected primers. Multivariate analysis placed some progeny as most similar to their parent inoculum genotype but others as less than 50% similar. This differs from published studies. Inocula comprising an equal mixture of a low and a high virulence population produced genotype profiles that did not resemble their parent inocula or one another. This was contrary to previous studies in other countries and suggested genetic reassortment or a very high mutation rate or both. The lack of stability through plant passage poses problems; not only does it call into question the value of genotyping but it also makes breeding for resistance to *P. brassicae* very difficult if the amount of polymorphism with microsatellite and RAPD primers reflects that of virulence genes, which not necessarily the case.

Differences from generation to generation in galls from field populations may be expected, but single-spore isolates (SSIs) should theoretically be genetically uniform. In Chapter 5, this was tested with four SSIs from the ‘e’ series (e1, e2, e3 and e6). Two accessions of each imported several years apart were not genetically identical and showed high polymorphism, some more than others. The e3 SSI was followed through three generations and, like field populations, showed high divergence from parent profiles. These unexpected results are difficult to explain unless the rate of random mutation is very high or the primary zoospores used to generate the SSI were binucleate. This casts doubt on the assumption that they are genetically homogeneous and so used to study events in infection and gall development.

In Chapter 6, the infection process was compared microscopically in susceptible and resistant host plants (cabbage and Chinese cabbage) growing in a hydroponic system and inoculated with two populations of *P. brassicae* with different degrees of virulence (low and high). Life cycle stages and host cell changes (hypertrophy and hyperplasia) were as described previously in other species with non-Australian pathogens. Root hair infection showed no

differences with pathogen virulence or plant resistance, as found by others, but secondary cortical infection did. The proportion of cells infected was greater with greater pathogen virulence. In infected cells, resting spores predominated in susceptible plants, whereas plasmodia predominated in resistant or partially resistant plants, as in other species, and it appears that the completion of the life cycle of *P. brassicae* is delayed in resistant and partially resistant plants, as suggested in other species.

An understanding of the genotypic and phenotypic diversity, genetic stability and infection processes of the pathogen populations are important for the development of *Brassica* cultivars with effective and durable resistance to this disease. This thesis provides new evidence of high genotype diversity in Australian *P. brassicae* that is not constant through plant infection. Unexpectedly this applied even in four SSIs from the ‘e’ series, also a new observation. It also shows that the critical difference between resistant and susceptible plants is the interruption of the *P. brassicae* life cycle at the secondary plasmodial stage in the host and so supports other previous research.

Chapter.1 General Introduction and review of the literature

1.1 Introduction

Plant diseases are responsible for losses of up to 10% of global food production and are a significant threat to global food security (Strange 2005). The annual cost of these losses is estimated at US$220 billion (Agrios 2005). In addition, plant pathogens are responsible for a range of additional economic, environmental and social costs including the cost of disease control and the impacts on environmental and human health of chemical control options.

In Australia, plant diseases cause significant losses in yield and quality of primary production in natural and managed systems (Chakraborty. S 1998). The economic impact of disease resulting from losses in productivity, the cost of disease management, and the economic penalty paid, for having to grow less profitable alternative crops or market lower quality produce, is high (**Table 1.1**). A large part of the losses in crucifer vegetables is due to *Plasmodiophora brassicae* Woronin (Woronin 1878).

Table 1.1: Losses in production of selected Australian agricultural commodities due to plant diseases (from Chakraborty et al. 1998).

Crop	Production (million tonnes)	Gross value ($ million)	Diseases loss ($ million)
Wheat	14.6	2901	438
Other grains	10.6	2532	259
Fruits	2.06	1653	245 (estimated)
Grapes	1.1	661	116
Vegetables	-	1107	166 (estimated)
Sugar	5.4	2100	67

The taxonomy of *P. brassicae* is confusing and constantly changing, mainly due to an incomplete understanding of the life cycle of this organism, and is discussed fully in Section 1.2.7. Briefly, historically the plasmodiophorids, including *P. brassicae*, were listed among the primitive fungi, but (Braselton 1995) placed the pathogen under Protista and the phylum Plasmodiophoromycota. The Kingdom Protista is no longer recognized because it was recognised as paraphyletic. Molecular investigation based on actin and ubiquitin proteins supported a cercozoan/foraminiferan ancestry for the plasmodiophorids (Archibald and Keeling 2004). Based also on the amoebidal nature of the plasmodium and flagellate

zoospores, these authors suggested that *P. brassicae* is in the phylum Cercozoa, which belongs to the kingdom Rhizaria.

P.brassicae continues to spread; it can be found worldwide in all temperate zones and infects over 64 genera of both cultivated and wild crucifers (Dixon 2009a). Symptoms of clubroot include swollen and distorted roots, and a reduction in growth or total failure of the root. This leads to wilting, a decrease in plant vigour, a reduction in yield and quality of *Brassica* crops and, in severe cases, plant death (Grabowski and Hopen 1985). The wide range of economically important hosts of *P. brassicae* includes *Brassica oleracea* (cabbage, cauliflower, broccoli, Brussels sprouts), *B. rapa* (turnip, turnip rape, Chinese cabbage and a range of leafy Asian greens), *B. napus* (swede, oilseed rape and fodder rape) (Dixon 2009a).

On average approximately 11% of *Brassica* and related crops globally are infected by clubroot (Dixon 2009a). Clubroot causes an estimated £30 million in crop losses each year in the UK (Stewart 2008). In Australia, clubroot disease is estimated to be responsible for crop losses of at least 10% annually and consequent loss of profits may reach up to AUD$16 million (Faggian 1999).

P. brassicae produces long-lived resting spores. Surrounded by a thick cell wall made up of chitinous and proteinaceous layers, these resting structures are highly resistant to biological and environmental degradation and are reported to persist in the soil for at least 15 years (Karling 1968, Mattusch 1977, Faggian 1999). The lifecycle of *P. brassicae* consists of two important phases: a primary phase that is restricted to root hairs and epidermal cells of the infected plants, and a secondary phase that occurs in the root cortex, causing abnormal root development (Ingram and Tommerup 1972).

Infected roots eventually disintegrate in the soil, releasing masses of spores; these long-lived spores can be transported in any manner that transports soil through the field. Soil can be carried on farm equipment, in water runoff and in dust by wind. Although the disease is not seed-borne, it is readily carried on infected transplants; however, Rennie et al. (2011) suggested that the dissemination of *P. brassicae* as external seed contamination is possible. Research indicates that infestations nearing 100% cause about 50% yield loss, while infestations of 10- 20% lead to 5- 10% yield loss. Further losses are due to a reduction in quality of vegetable and oil seed crops, as in canola (Pauly 2009).

Despite the fact that this organism causes such large economic losses and has been widely researched for many years, there are still significant gaps in the knowledge of the biology (its

infection cycle and diversity) of *P. brassicae* and the management and control of clubroot disease. An improved understanding of the cellular and molecular interactions between host plant and pathogen at different steps of the infection cycle will help to identify potential targets for developing resistance in important *Brassica* crop species (Agarwal 2009). The challenge remains to more fully understand the behaviour of the pathogen on a genomic and molecular level and to use this information to improve control of this pathogen. (Howard, Strelkov et al. 2010).

1.2 Clubroot disease

1.2.1 Local (vernacular) names of clubroot

Historical records indicate that infection originated in Europe. Towards the end of the 19th century clubroot, known then as 'finger and toe' because of the shape of infected roots, caused a severe epidemic in St. Petersburg. The causative pathogen was identified by a Russian scientist named Mikhail Woronin in 1875. The disease is also known by a range of vernacular names, some of which are descriptive (e,g. finger and toe, and ambury or anbury which means soft tumour) or reflect the local importance of crops affected (e.g. the German Kohlkropf, meaning cabbage disease and the French maladie du chou, meaning disease of cauliflower) (**Table 1.2**) (Dixon 2009a).

1.2.2 Clubroot around the world

One of the earliest reports of clubroot-like symptoms, 'paleness of affected plants and spongy roots of rape turnip and radish grown in soil fertilized with manure', was from Italy in the 4th century AD (Watson 1969), making clubroot one of the oldest documented plant diseases and likely to have affected crops such as turnips cultivated by the Romans for feeding humans and animals. It is believed that cattle dung may have been an early means of spread of the pathogen (Howard, Strelkov et al. 2010).

Buczacki (1985) states that Diaz de Isla in Spain (1539) described infection on cabbages as clubroot symptoms (Howard, Strelkov et al. 2010). He observed a huge spherical gall on the roots of *B. oleracea (*cv. Premium Crop), further supporting the assumption that clubroot originated in the Mediterranean region, not far from the centre of origin of the genus *Brassica* (Dixon 2009a). Officially Ellis (1750) documented the first record of clubroot disease in England (Buczacki 1985). This was followed by a series of reports from across Europe in the

Table 1.2: Common names for clubroot disease in different countries (from Dixon 2009a).

Country	**Common names**
Australia, New Zealand	Clubroot
Belgium	Bosse, gross pied, kanker, klinger, knobbel, knoll, knoop, knotze, knuist, kwab,kwabbe, kwabbel, kwabbeziekte, kwadevoeten, oolen, tol, wratten, verrue
Denmark	Kaalbrok, kaolbrok
Finland	Mohojuuri
France	Hernie du chou, gros pied, maladie digitoire, maladie du chou
Germany, Switzerland, Austria	Fingerkrankheit, galle, herniekrankheit, huas, kelch, klumpenfuss, knotesucht, kohlhernie, kohlkropf, krof des kohles, kropfkrankheit des kohles, kuss, nolle,
Great Britain	Club root, finger-and-toe, anbury, banbury, clubbing
Italy	Ernia, mal degosso dei cavoli, tuberculosi dei cavoli
Netherlands	Knolvoet
North America	Club root, club foot, clump foot, finger-and-toe,
Norway	Klumprot
Russia	Kapoustnaja
South Africa	Club root, finger and toe, club foot, dik voet
Spain	Hernia, hernia de la col, potra
Sweden	Klumprotsjuka

following century confirming the appearance and widespread distribution of clubroot disease. The use of root crops such as turnip and other cruciferous plants as popular foods in many countries, together with the increasing intensity and frequency of crop production needed to feed burgeoning populations serving the industrial revolution, encouraged the spread and proliferation of *P. brassicae* and increased the severity of infestation and symptom expression. Clubroot has become a significant problem in Russia, Canada, Northern Africa, Northern and Southern America, Australia, New Zealand, Japan, Hawaiian Russia and New Zealand (Mazin V.V. 1976, Voorrips 1995, Tewari 2005)(**Fig. 1.1**).

In Canada, clubroot field surveys confirmed that clubrooot continues to be a problem in many regions of Alberta and that moreover it was spreading; out of 18 counties surveyed, 566 clubroot-infested fields were recorded (Strelkov et al. 2011).

Russian cabbage crops were heavily infected by *P. brassicae* during the 1860s and incurred large yield losses. Woronin provided a full description and identified the main cause of clubroot disease (Woronin 1878). In 1853, clubroot was recorded for the first time in North America (Watson 1969).

The disease is becoming increasingly important in emerging economies like India and has been recorded in neighbouring countries such as Nepal since 1993 (Cicu 2006). Widodo and Suheri (1995) reported that widespread outbreaks of clubroot had occurred in many provinces, causing damage to 88.6% of total cultivated cabbage crops. In Japan, clubroot was described for the first time in 1982 (Naiki 1987). Nakata and Takimoto (1928) published the first report of clubroot on Chinese cabbage in Korea in 1928. Kim et al. (2002) added that the disease had become severe in alpine areas of Kyonggi and Kangwon, gradually spreading to plains fields throughout the country. Clubroot has remained the greatest factor limiting production of Chinese cabbage since the 1990s. Clubroot is considered a serious disease in Canada and Europe, where estimated annual losses from canola plants of approximately 1.6 t/ha are reported (Hind-Lanoiselet and Parker 2005).

Figure 1.1: The distribution of clubroot disease around the world. The green dots represent countries where clubroot has been reported.

http://www.plantwise.org/KnowledgeBank/Map/GLOBAL/*Plasmodiophora_brassicae*/

Fodder turnips imported as a feed source for livestock by early settlers is thought to have been an important means of spread of the pathogen into countries such as Australia and New Zealand. Clubroot is currently widespread in most vegetable production regions of Australia and has been reported in most Australian states; Queensland (Simmonds 1966), South Australia (Cook and Dubae 1989), Tasmania (Sampson and Walker 1982), Victoria (Washington and Nancarrow 1983) and Western Australia (Shivas 1989) (**Fig. 1.2**). To date the disease has not caused any significant problem in the Australian canola crop although, based on recent Canadian experience, it is a significant risk (Donald et al. 2006b; Donald and Porter 2010b; Khangura and Wright 2012). Hind-Lanoisele and Parker (2005) note that, with the exception of Tasmania and some parts of NSW where disease occurs year round, most Australian pathotypes of *P. brassicae* only cause disease in the warmer months and irrigation water is required for dispersal. In Victoria, more than 70% of farms are affected, with the estimated annual cost to control clubroot reported to be in excess of AUD$500 ha^{-1} by 28% of growers on affected properties (Donald et al. 2006a).

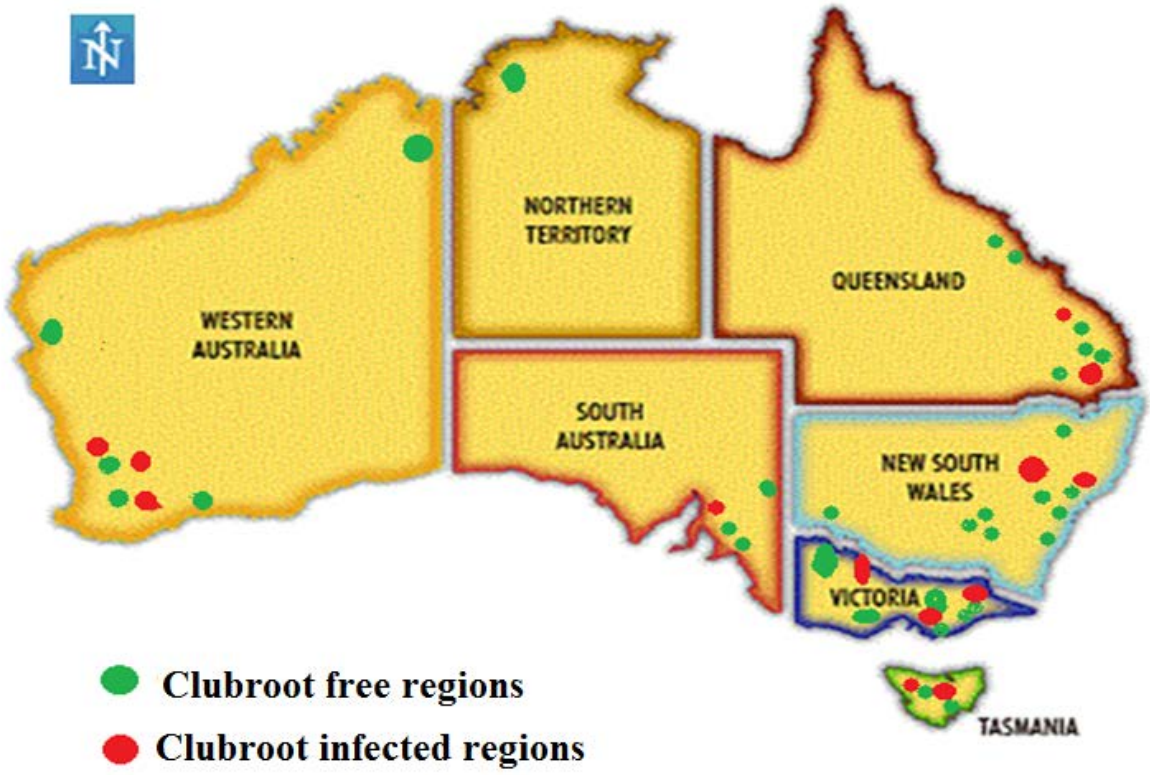

Figure 1.2: Distribution of clubroot in different states in Australia (Source: Shivas 1989).

According to reports by the Commonwealth Mycological Institute (CMI) (1977) and the European Plant Pathology Organisation (Dixon 2009a), clubroot is recorded as present or widespread in 90 countries around the world. Clubroot remains unknown in many countries, in particular undeveloped countries, with simplicity of diagnosis and poor education contributing to a lack of knowledge about the disease (Dixon 2009a).

Detailed research has been conducted in Great Britain, Germany, the Netherlands, the United States of America, Canada and Australia, where clubroot continues to cause significant losses in vegetable and oilseed brassica hosts.

1.2.3 Host range

Plasmodiophora brassicae can infect and cause symptoms of disease in members of the family *Brassicaceae.* There are over 330 taxa in about 61 genera and 3700 species of crucifers, including weeds, that are potential hosts for *P. brassicae* (Karling, 1968; Ludwig-Muller et al., 1999b; Dixon, 2009a) (**Table 1.3**) with cabbage being one of the crops most susceptible to clubroot (Sherf and MacNab, 1986). Economically important host crops include the vegetable crops broccoli, cauliflower, cabbage, Chinese cabbage, Brussels sprouts, radishes and a range of leafy Asian greens and oilseed and fodder brassicas, canola, mustards, fodder rape and fodder turnip. Many weeds in the Brassicaceae are also susceptible, e.g. Shepherd's purse (*Capsella bursa-pastoris*) (**Table 1.4**). These are generally considered important as alternative hosts that contribute to the survival of *P. brassicae* in infested fields in the absence of cruciferous crops (Halsted 1894; Karling 1968; Tanaka et al. 2006). However, recent epidemiological studies indicate that the relationship between *P. brassicae* populations from cruciferous crops and weeds may be not as simple as first thought (Osaki et al. 2008b).

While symptoms of clubroot are unique to members of the *Brassicaceae*, *P. brassicae* is capable of infecting the root hairs of the following non-cruciferous hosts and others (**Table 1.5**): Poaceae [creeping bent grass (*Agrostis alba* var. *stoloniferae*), orchardgrass (*Dactylis glomerata*) and ryegrass (*Lolium perenne*)]; *Rosaceae* [strawberry (*Fragaria* spp.)]; *Papaveraceae* [corn poppy (*Papaver rhoeas*)]; *Polygonaceae* [dock (*Rumex* spp.)]; *Resedaceae* [mignonette (*Reseda odorata*)]; and *Fabaceae* [red clover (*Trifolium pratense*)] (Webb 1949; Macfarlane 1952 as stated by Ludwig-Muller and Schuller, 2008; Donald et al. 2006a; Kim et al. 2010).

Table 1.3: Examples of clubroot-susceptible host plants (from Donald and Porter, 2010a)

Degree of susceptibility	Host plants
Most susceptible	Cabbage, Chinese cabbage, Brussels sprouts, some turnips, wormseed mustard and some species of candytuft
Medium susceptible	Kohlrabi, kale, cauliflower, collards, broccoli, rutabaga, seakale, some turnips and radishes and some species of candytuft
Mildly susceptible	Rape(seed) (canola), black mustard, some turnip and radish varieties and tumble mustard
Very resistant or tolerant, occasionally immune	Wintercress or yellow rocket, horseradish, shepherd's purse, wallflower, dame's violet, peppergrass, garden cress, stock and some radishes

Table 1.4: Common cruciferous weeds that are susceptible to *P. brassicae* (from Donald and Porter, 2010a)

Botanical Name	Common name
Brassica campestris (L.) A. R. Clapham	Bridal rape
B. tournefortii. Gouna	Wild turnip
Capsella bursa-pastoris (L.) Medikus	Shepherd's purse
Conringia orientalis (L.) Dumort	Wild cabbage
Diplotaxis tenuifolia (L.) DC	Sand mustard
Lepidium campestre (L.) R.Br	Field cress
Myagrum perfoliatum L.	Musk weed
Raphanus raphanistrum L.	Wild radish
Raphanus rugosum (L.) All.	Turnip weed
Sinapsis arvensis L.	Charlock
Sisymbrium officinale (L.) Scop.	Hedge mustard
Vella annua L.	Ward weed

Table 1.5: Non-cruciferous plants that are susceptible to infection by *P.brassicae* (from Donald and Porter, 2010a)

Botanical Name	Common name
Agrostis alba L. var. *stolonifera*	Creeping bent grass
Dactylis glomerata L.	Cocksfoot
Fragaria sp.	Strawberry
Holcus lanatus L.	Yorkshire fog grass
Lolium perenne L.	Perennial ray grass
Mathiola incana (L.) W.T.Aiton	Stock
Papaver rhoeas L.	Flanders poppy
Reseda odorata L.	Common mignonette
Rumex spp.	Dock
Tropaeolum majus L.	Nasturtium

1.2.4 Symptoms

Disease symptoms can vary slightly depending on the host plant and the severity of the disease (related to the degree of pathogen virulence and aggressiveness) (**Table 1.6**). Severely infected roots have stunted growth, leaf chlorosis (blue-purple tinge to yellow) and wilting of leaves during warm days. Infected galls appear, develop a brown colour and slowly disintegrate in soil, causing the whole plant to die. Severely infected plants that do survive for the entire life of the crop are unlikely to produce marketable produce (Kong Kaw Wa 2009).

In the field the first obvious symptom of disease is wilting of the foliage, especially on hot and sunny days, and partial recovery of turgidity during the night (Agrios 2005; Kong Kaw Wa 2009). Wilting is due to the uncontrolled swelling and cell divisions (hypotrophy and hyperplasia) and splitting of the vascular cylinder by infection and enlargement of parenchyma. The resulting enlarged roots appear spindle-shaped, knobby, spherical or club-shaped. All roots, including the underground part of the stem (hypocotyl) may be affected. These malformed and greatly enlarged roots (**Fig. 1.3**) are the key symptom of this disease (Karling, 1968). Impaired roots have a reduced capacity to absorb water and nutrients and so the foliage may appear yellowish; the plant may be stunted and its yield reduced (**Fig. 1.3**). In some infected plants, increased branching of shoots and roots and production of leaf-like teratomas occurs. Severe stunting and even plant death may be evident if infection occurs

early and the disease progresses rapidly (Brown and Morra 1997; Agrios, 2005). In severe cases, entire plantings are destroyed. Eventually root galls are invaded by secondary parasitic or saprophytic microorganisms, causing them to decay, disintegrate and release resting spores into the soil (Ito et al. 1999a). However, there is no evidence that these organisms are involved in the infection and development of *P. brassicae* (Karling, 1968).

Figure 1.3: Symptoms of infection by *Plasmodiophora brassicae*. Field infected with *P. brassicae*, plants stunted and discoloured (1), wrinkled leaf symptom (1 inset), symptoms in host plants Chinese cabbage (*Brassica rapa* var. *pekinensis* Lour.) (2), broccoli (*Brassica oleracea* var. *italica* Plenck) (3), turnip (*Brassica rapa* var. *rapa* L.) (4), and rutabaga (*Brassica napus* var. *napobrassica* (L.) Rchb.) (5) (Source of images "2; 3; 4 and 5" University of Georgia Plant Pathology Archive. (2010).

Table 1.6: Clubroot disease symptoms on *Brassica* crops (Zitter 1985; Donald and Porter 2003b; Agarwal 2008).

Type of brassica crop	**Common disease symptoms**
Asian vegetables including bok choy, Chinese chard, Chinese flowering cabbage, mustard green	Wilting leading to complete collapse, premature death of severely affected plants, club-shaped roots.
Broccoli (*B. oleracea* L.var. *italica*)	Wilting, stunting, leaves chlorotic or with blue-purple tint, club-shaped roots, formation of small poor-quality head.
Cabbage (*B. oleracea* L. var. *capitata*)/ Brussels sprouts (*B. oleracea* L. var. *gemmifera*)	Wilting, stunting, leaves chlorotic or with blue-purple tint, club-shaped roots, small losses, hearts of poor quality.
Canola/oilseed rape (*B. napus* L.)	Infection at the seedling stage: wilting, stunting and yellowing symptoms by the late rosette to early podding stage. Infection at late stage: plants ripen prematurely, seeds shrivelled, reducing yield and quality (oil content), club-shaped roots.
Cauliflower (*B. oleracea* L. var. *botrytis*)	Wilting, stunting, leaves chlorotic or with blue-purple tint, club-shaped roots, severely affected plants do not produce marketable heads.
Chinese cabbage (*B. rapa* L. var. *pekinensis*)	Wilting leading to complete collapse, premature death of severely affected plants, club-shaped roots.
Radish (*Raphanus sativus* L.), Turnip, swede (*B. rapa* L. var. *rapa*)	Wilting chlorotic leaves or with blue-purple tint, mis-shapen roots.
White mustard (*Sinapsis alba* L.)	Wilting, stunting, leaf lesions symptomatic of nutrient deficiency, root galls ranging from tiny nodules to big club-shaped overgrowths.

1.2.5 Disease Life Cycle

To understand the behaviour of a microorganism and look for methods to control it, the fundamental first step should be a solid understanding of the life cycle and infection process. In the past 50 years many researchers have sought to understand the life cycle of and to control *P. brassicae* infections (Macfarlane, 1952; Karling, 1968; Ayers 1944; Ingram and Tommerup, 1972; Naiki, 1987; Buczacki and Cadd, 1976; Buczacki and White, 1979). In spite of this, much of the life cycle remains 'poorly understood' (Donald et al. 2008).

P. brassicae is a soil-borne obligate protist. There are three important stages during its life cycle as reviewed by Kageyama and Asano (2009): resting spores and their survival in soil; the release of primary zoospores from resting spores and their subsequent infection of root hairs; and fusion of two distinct zoospores to form secondary zoospores that infect the cortex. Not all researchers agree with this sequence, however, as discussed later.

In brief, when field conditions are conducive, clusters of thick walled resting spores (**Fig. 1.4a**) are released into the soil when infected roots deteriorate and decay (Karling, 1968). Resting spores release biflagellate primary zoospores (**Fig. 1.4 and Fig. 1.5**). The flagella are anterior and unequal in length and shape, one being short with a blunt end and the other long with a whiplash or tail-piece (**Fig. 1.4b**) (Ayers 1944).

When a zoospore reaches the surface of a root hair, it encysts and begins to penetrate the cell wall of the host plants, initiating the primary infection stage (the root hair infection stage). In root hairs the pathogen forms primary plasmodia. These undergo simultaneous nuclear divisions before cleaving into zoosporangia (**Fig. 1.4c**). The zoosporangia form clusters in the root hairs (**Fig. 1.4d**) and sometimes in epidermal cells. Over time, 4–16 secondary zoospores are formed in each zoosporangium. After releasing these zoospores, the empty zoosporangia remain in the root hairs (**Fig. 1.4e**). The secondary zoospores cannot be visually differentiated from the primary zoospores. Binucleate zoospores have occasionally been reported and interpreted as having formed by the fusion of two distinct zoospores, not from division within nuclei as Tommerup and Ingram (1971) and Ingram and Tommerup (1972) documented in their studies. The secondary zoospores penetrate the cortical tissues to form the secondary infection stage (cortical infection) which takes place deeper in the cortex and stele of hypocotyl or root (Dixon and Page, 1998). Secondary plasmodia develop in the infected cells and proliferate. The secondary plasmodia contain two nuclei in the early stages of growth and

then develop after a number of nuclear divisions into multinucleate plasmodia (Garber and Aist 1979a & 1979b; Mitani et al. 2003). In plasmodia with haploid nuclei, the nuclei may fuse forming diploid nuclei. Alternatively, meiotic cleavage may occur in these diploid plasmodia, indicating that the plasmodia return to the haploid state again (Buczacki 1983a), although this is not universally agreed. Uncontrolled cell division (hypertrophy) and elongation (hyperplasia) results in the formation of root galls (**Figs 1.4f,g**) (Ingram and Tommerup 1972) containing resting spores, which are the long-term survival structures (**Figs 1. 4h,i**) (Ikegayami et al. 1982). Ingram and Tommerup (1972) reported resting spore germination and development into primary zoospores from 3–4 days after inoculation, followed by encystment on root hairs and penetration of the cell wall. In this study, primary plasmodia were observed in root hairs and epidermal cells at 7-13 dai. Interaction between the host and pathogen was observed at 10-23 dai by Siemens et al. (2006) using microarray technology but more recently, another microarray study showed that pathogen penetration occurred from day 4 onwards and then primary plasmodia developed within the host root (Agarwal et al. 2009).

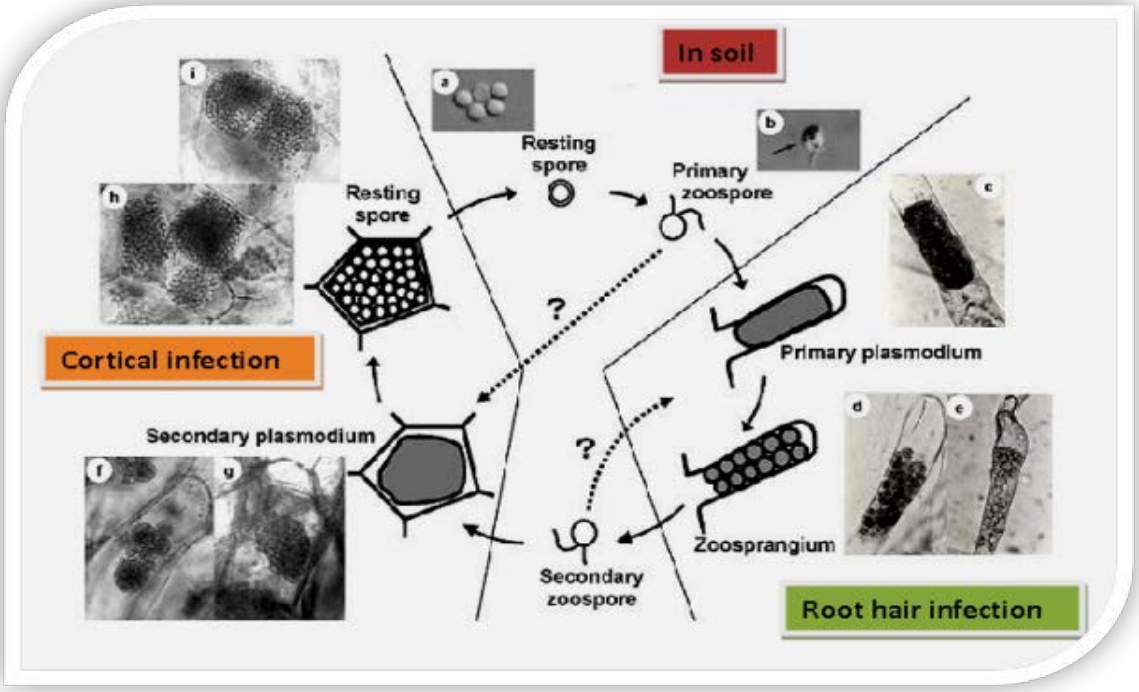

Figure 1.4: Life cycle of *Plasmodiophora brassicae*. a. resting spore. b. primary zoospore, c. primary plasmodium in root hair, d. zoosporangial cluster in root hair, e. empty zoosporangium, f,g. secondary plasmodia in cortical cells, h,i. resting spores in cortical cells (Kageyama 2009).

According to Devos et al. (2005), the pathogen undergoes a transition from the primary phase to the secondary phase at 13 dai. This research confirmed that the secondary phase of the life cycle occurred in the cortical cells after 13–15 days in soil-grown plants. During the infection process, the pathogen develops from the primary to the secondary phase after 13-15 days (Devos et al. 2005; Agarwal et al. 2009). The secondary plasmodia multiplied into resting spores within the cortical cells, proliferating into galls by 28 days.

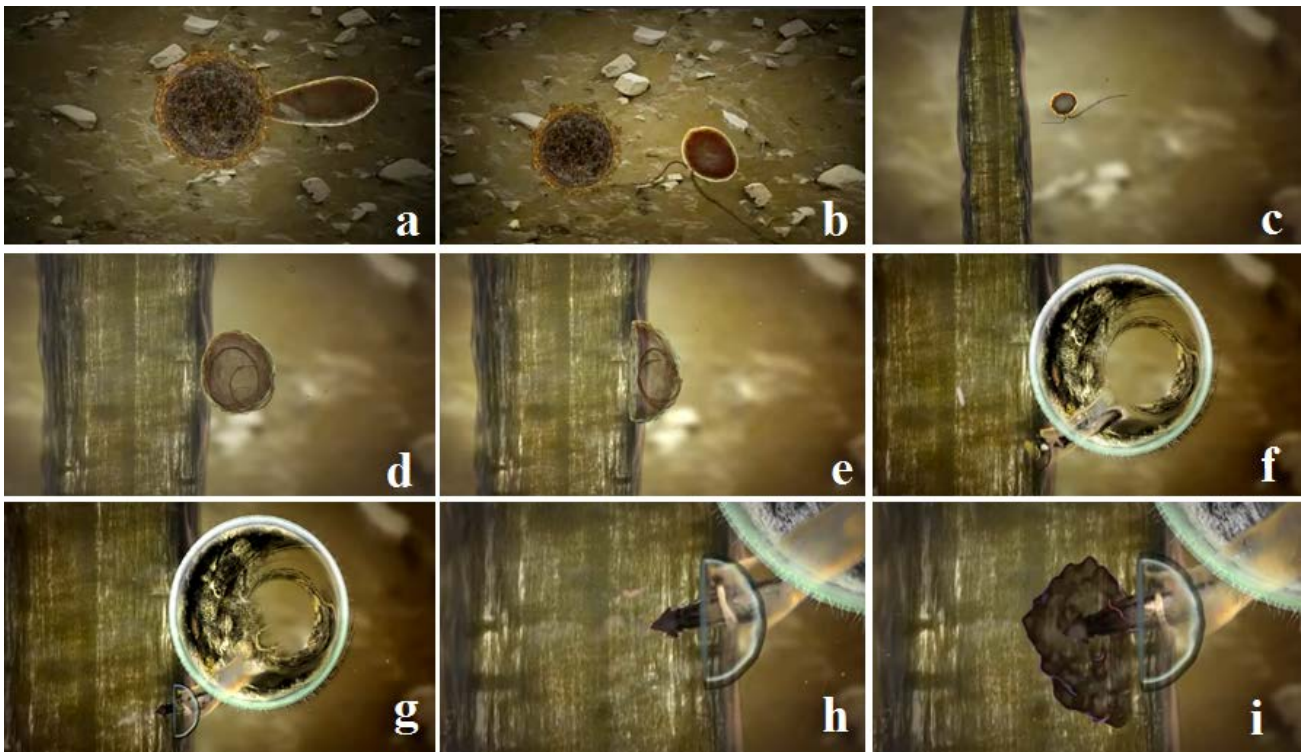

Figure 1.5: Orientation during zoospore encystment and cyst germination/infection events. (a) resting spore germination, (b) zoospore release, (c) swimming flagellate zoospore, (d) zoospore resting on cell surface, (e-h) penetration of cell wall by encysted zoospore (Canola Council of Canada 2013; http://www.youtube.com/watch?v=3dyQhsqIu0o&list=PL297C495671C5B2C1).

Agarwal et al. (2009) showed that disease expression was greater in plants grown in a growth cabinet than in plants grown in a glasshouse. This demonstrates the importance of optimum temperature and high humidity, which are best maintained in a growth cabinet. Controlled conditions together with the use of sand-liquid culture (Donald and Porter, 2004a) have greatly improved the ability of researchers to study the lifecycle of this pathogen and to determine the influence of modified environments on it. In spite of this, there is still debate about the transition between primary and secondary infection and no clear evidence that can

clarify the non-cruciform division through sporangial development and sporogenic plasmodia (Braselton 2005).

1.2.6 Plasmodiophora brassicae behaviour

P. brassicae is a biotrophic organism, and by definition cannot be grown in axenic artificial culture. Typical experimental systems required to study its behaviour and lifecycle are therefore more complex than for other, culturable organisms (Agarwal et al. 2009; Siemens et al. 2009a). Arnold et al. (1996) presented some evidence demonstrating a brief free-living saprotrophic amoeboid stage in the *P. brassicae* lifecycle. These authors further suggested culture in a model medium such as Ringer's agar containing a suspension of *Escherichia coli* but this method has not been widely used by researchers.

Mendge and Hahn (2002) proposed that biotrophic pathogens are able to inhibit host defences for at least part of the infection period. There is only limited information on the behaviour and the biotrophic lifestyle at the genetic and molecular level for pathogens such as *P. brassicae*, particularly when compared with necrotrophs.

Siemens et al. (2009a) emphasised that *P. brassicae* is an unstable pathogen, as stored spores may fail to infect host plants because of declining viability. Spore viability is dependent upon the age and condition of the spores at the time of collection and the conditions of storage. It is therefore essential that test conditions are standardised and adequate infection controls are included with susceptible lines in every test.

1.2.7 Taxonomy of Plasmodiophora brassicae

The taxonomy of *P. brassicae* is the subject of ongoing debate and constant change (Doland and Porter 2010b) and there is a broad discussion of the taxonomic status of this ‘evolutionary oddity’. The obligately biotrophic nature of *P. brassicae* makes it difficult to study its life history (Ingram and Tommerup 1972; Tommerup, and 1971 Ingram).

In 1972 Waterhouse placed the Plasmodiophorida in the Kingdom Fungi, as they produce spores, but Margulis et al. (1989) emphasised that they should be considered protoctists and placed them in the Plasmodiophorida in the Kingdom Protista. Plasmodiophorida contained a range of eukaryotic intracellular parasites, including several serious pathogens affecting a large number of economically important agricultural crops and others that are vectors for several plant pathogenic viruses (Braselton 1995; Bulman et al. 2006).

P. brassicae was also classified in the Eukaryota in the Protozoa (Barr 1992; Braselton 2000) (**Fig.1.6**). Numerous efforts have been devoted to clarifying the important characteristics for classification of *P. brassicae* (Barr, 1992; Archibald and Keeling, 2004; Braselton, 2005).

Braselton (1995) described plasmodiophorids as monophyletic with unconfirmed systematic relations. Braselton (2001) included two pathogens in the order Plasmodiophorida. The first of these was *Spongospora subterranea*, which causes powdery scab disease in potato. The second was *P. brassicae,* the causal agent of clubroot in cruciferous plants.

Characteristic features of the plasmodiophorids include: (i) their ability to reproduce through a form of closed mitosis known as cruciform nuclear division, the nucleolus of the cell having a cruciate appearance during the metaphase stage, (ii) zoospores with one long and one short anterior whiplash flagellum, and (iii) the 'Rohr and Stachel' structures used by the pathogen to penetrate the host plant cells in the same way a nematode might use its stylet. Other key features of plasmodiophorids include an environmentally resistant structure called a resting spore (formerly known as a cyst), a multinucleate protoplast (commonly referred to as a plasmodium) and their ability to live as obligate intercellular parasites. Plasmodiophorids have two major life cycle phases: the primary phase (sporangial or sporangiogenous) and the secondary phase (sporogenic or cystogenous). Each phase begins when a single, uninucleate zoospore infects a host cell (Braselton 1995). The most important pathological symptoms after the invasion and infection by plasmodiophorids are the occurrence of hypertrophy and hyperplasia in the infected plant tissue and the emergence of galls in the root zone.

Plasmodiophorids have complex and incompletely understood life cycles, consisting of a resting spore, primary zoospore, primary plasmodia, secondary zoospore, multinucleate secondary plasmodia and sporangial stages. These characteristics have made it difficult to position these organisms in terms of eukaryotic evolution. The group has been placed between the most primitive fungi as modern representatives of the most advanced fungal ancestors (Buczacki 1983b; Braselton 1995). Evolutionary trends and relationships are usually determined through the phylogenetic analysis of small subunit ribosomal RNA (rRNA) genes. Ward and Adams (1998) demonstrated that with respect to rRNA, plasmodiophorids are not true fungi as they do not group convincingly with the fungi or with any other groups of micro-organism. Cavalier-Smith and Chao (2003) later proposed classification of the plasmodiophorids in the Kingdom Protozoa in the Phylum Cercozoa. Ultimately, as a result of analysing the sequences of the proteins actin and ubiquitin,

Archibald and Keeling (2004) determined that plasmodiophorids were most closely related to the Cercozoa (**Fig. 1.6**).

- **Domain *Eukaryota***
  - **Kingdom Protozoa**
    - **Subkingdom *Biciliata***
      - **Infra-kingdom *Rhizaria***
        - **Phylum "Obligate intracellular parasites" Cercozoa .**
          - **Subphylum "Endoparasitic slime molds" Endomyxa**
            - **Class *Phytomyxea***
              - **Order *Plasmodiophorida***
                - **Family *Plasmodiophoridae***
                  - **Genus *Plasmodiophora***

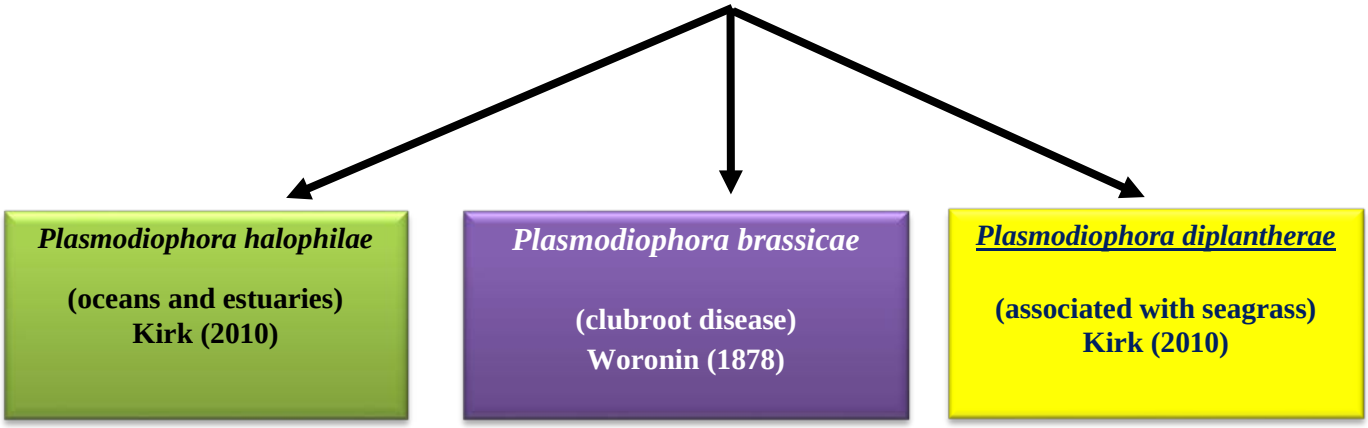

Figure 1.6: Taxonomy of *Plasmodiophora brassicae* (from Olive 1982; Braselton 2000; Brands 2005).

1.3 Infection process

The infection process in clubroot disease of cabbages has been studied by many researchers (Monteith 1924; Naumov 1927; Wellman 1930; Gibbs 1931; Griess et al. 1944; Macfarlane 1952; Colhoun 1953) who sought to use this knowledge to reduce the impact of the disease. In most instances, the simple way to achieve this has been by growing plants in naturally and artificially contaminated soils under varying conditions of moisture, temperature, spore load, soil pH and lime content to determine the response of the pathogen (Colhoun 1953).

1.3.1 Changes in the host upon infection

As the pathogen invades its host, colonizing plant cells, changes occur in the host on a number of levels. Although many facets of the interaction of *P. brassicae* with its hosts are yet to be fully elucidated (Graveland et al. 1992), key changes are summarised below and discussed more fully in Chapter 6.

A. Morphological changes

Enlargement and deformation of infected roots is the most visible symptom of infection. This is a result of the enlargement of infected cells and the occurrence of hypertrophy and hyperplasia, key characteristics of clubroot in the infected tissues (Jubault et al. 2008; Ludwig-Muller et al. 1994b).

B. Anatomical changes

Ludwig-Muller et al. (1994b) state that *P. brassicae* appears first in the epidermal and then cortical parenchyma and subsequently infiltrates, stimulating changes in cambium and medullary rays. The processes of cell enlargement and DNA stimulation initiate cell division. Infected cells increase in size, becoming about 20-30 times bigger (hypertrophy), and multiply (hyperplasia), with continuous mitosis occurring after cytokinesis has ceased. This is accompanied by an increase in the volume of the nucleus and an increase in the number of nucleoli (Brown and Morra 1997).

Kim and Lee (2001) investigated the infected roots of Chinese cabbage (a common susceptible host plant) after colonisation by *P. brassicae*. Light and electron microscopy showed histopathological changes as a result of invasion. Cross sections indicated the presence of resting spores in the cortex and partly in the stele, invading the xylem (**Fig. 1.7**).

As cells were infected, one to several plasmodia were observed. Organelles like dictyosomes and mitochondria increased and the cytoplasm became more dense with a central vacuole. As infection progressed, uninucleate and multinucleate plasmodia were observed. These contained mitochondria, lipid droplets, endoplasmic reticulum and sometimes small vacuoles. In addition, chains of spherical vesicles were formed in uninucleate plasmodia as a result of the degeneration of plasmodial fragments (Kim and Lee, 2001).

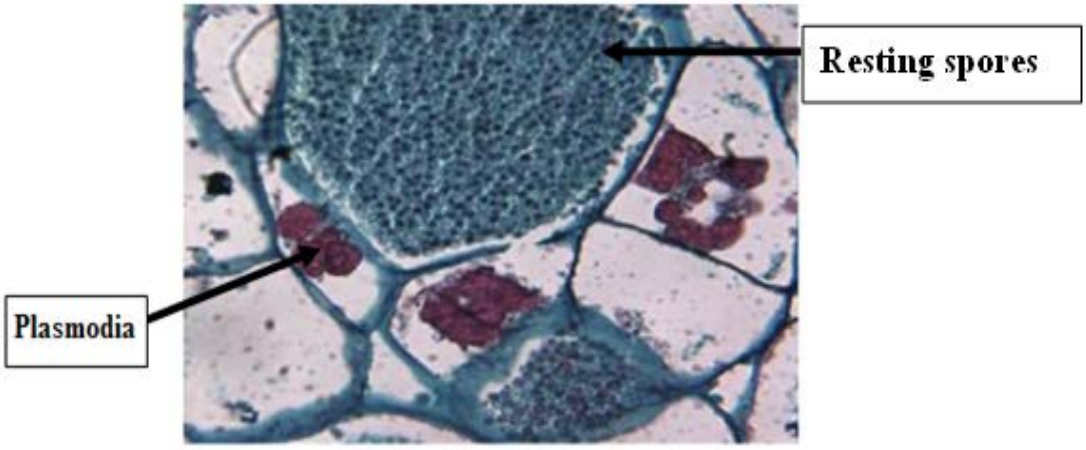

Figure 1.7: Infected cells of host plants filled with resting spores or plasmodia.

Graveland et al. (1992) explained that differences in visual symptoms of disease can be attributed to the different patterns of cell division. A symmetrical pattern leads to the thickening of a relatively long piece of root and a more disorganised pattern of division leads to galls. Aist and Williams (1971) noted the deposition of callose between the host plasma membrane and the cell wall at the penetration site of the pathogen in the host root hairs.

A number of researchers have studied differences in infection patterns in resistant versus susceptible host plants (Dekhuijzen 1976a; Gunnarsson et al. 1986; Gustafsson and Falt 1986; Kobelt et al. 2000; Andrea et al. 2000; Donald et al. 2008). In each case, infection occurred in both resistant and susceptible hosts but with varying degrees of symptoms, e.g. the evolution of the plasmodium through the various stages of infection. The fundamental difference between resistant and susceptible hosts occurs in the xylem (Kobelt et al. 2000; Andrea 2000; Donald et al. 2008); resistant hosts have no degradation of the secondary thickening and cell walls of the xylem (**Fig. 1.8**). Moreover, in resistant hosts the pathogen is restricted to a limited number of infected cells; also Vasil and Vimla (1987) noted that infected cells are sometimes bounded by necrotic cells. In some instances hypertrophy and hyperplasia resulted in a small number of spores being produced together, with the formation of mini-galls.

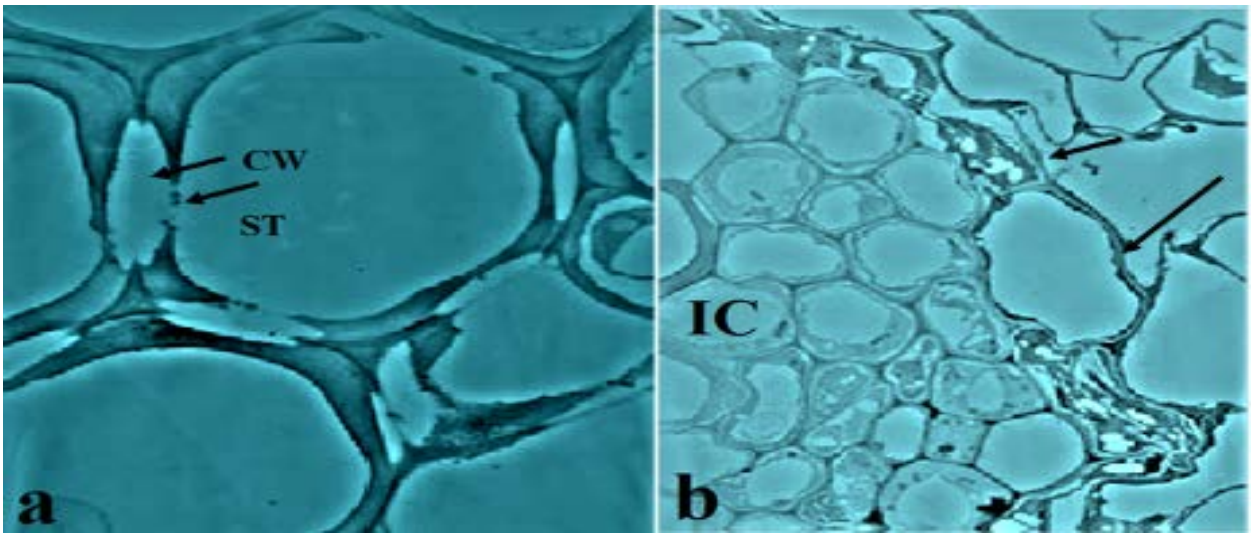

Figure 1.8: Transmission electron micrographs of (a) the susceptible and (b) resistant reaction in inoculated cauliflower roots. The susceptible reaction features broken cell walls (CW) and degradation of the secondary thickening (ST) of xylem cells. The susceptible reaction features a healthy inner cortex (IC) surrounded by disorganized, infected cell layers (arrows). Images courtesy of Dr Caroline Donald (Department of Primary Industries Victoria, Australia).

After successful invasion and during the secondary infection process, no evidence of an induced defence reaction is reported, nor are necrotic responses evident. The enlarged host nucleolus appears to be related to the maintenance of a meristematic condition, which is a key response of the host cell to the pathogen. Moreover, the intact outer membrane of the plasmodial envelope may be under host control, since its degeneration occurs at the same time as host ribosomes break down (Williams and Mcnabola, 1967).

In heavily infected roots the host appears to lose control of its cells, with pronounced cell enlargement and cell proliferation occurring in most infected cells. Growth of leaf-like swellings from the roots is a result of the plant hormones auxin and cytokinin being involved in disease development (Ludwig- Muller and Schuller, 2008).

Studying *Arabidopsis thaliana*, Siemens et al. (2006) noted two critical events in the infection cycle. The first occurred 8-10 dai. At this time small secondary plasmodia of *P. brassicae* could be observed; approximately 20% of the host tissue was colonized but there was little change in host root cell morphology. Later, at 21-23 dai, numerous stages of the

pathogen were visible within host tissue; approximately 60-65% of the host root cells were colonized and the root morphology was completely changed.

C. Molecular level

Infection by the pathogen leads to changes in the metabolism of its hosts. Changes in the chemical composition of the host tissue can influence the diversity of the forms that feed on its tissues and on the production of resting spores (Neuhauser et al. 2011).

Hormonal imbalance is accompanied by phenotypic changes. Ludwig Muller and Epstein (1994a; 1994b) noted that symptoms appearing as a reaction to the invasion of the pathogen into the host plant tissue appear firstly in the host cortical parenchyma and then spread, stimulating changes in the vascular cambium and medullary rays. This results in an increase in the activity of hormones like indoleacetic acid (IAA), which stimulates the cells to increase in size (hypertrophy).

In the host tissue are many complex substances that control development. Abscisic acid (ABA), ethylene, jasmonic acid (JA), and salicylic acid (SA) are plant hormones that play important roles during abiotic and biotic stress signalling (Ludwig-Muller and Schuller, 2008). Siemens et al. (2006) used reporter genes in *A. thaliana* to localize auxin and cytokinin responses to the part of the root where gall formation occurs. Devos et al. (2006) showed that both host and pathogen produce hormones that increase expansion and cell division during infection. In particular, in early stages of infection, infected plant cells produce cytokinins. This leads to the formation of meristematic regions in the root cortex, as a result of cell division, and triggers gall development. Meristematic cells act as sinks for carbohydrates and amino acids, which sustain the pathogen development.

Transcriptome analysis of *A. thaliana* plants infected by *P. brassicae* indicated that genes associated with cytokinin homeostasis (cytokinin synthase *IPT3*; cytokinin oxidase/dehydrogenases *CKX1* and *CKX6*) were strongly down-regulated early in pathogen colonization (Siemens et al. 2006). The expression of numerous genes encoding proteins involved in cytokinin signalling was also influenced. Furthermore, transgenic plants over-expressing cytokinin oxidase/dehydrogenases (CKX1 and CKX3) were disease-resistant, showing the importance of cytokinins as a key factor in clubroot disease development.

Cytokinins are a class of plant hormones that were first identified as factors promoting cell division during the 1950s and 1960s (Miller et al. 1955; Letham 1963, cited by Husickova 2010). Cytokinins are N6-substituted adenine derivatives involved in the control of several important processes related to plant growth and development. They play important roles in the control of cell division, chloroplast development, bud and root differentiation, shoot meristem initiation and growth, stress tolerance and senescence (Mok and Mok 2001; Schmulling 2003; Werner and Schmulling 2009). Cytokinins also encourage the differentiation of plastids into chloroplasts and increase the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase and NADP-glyceraldehydephosphate dehydrogenase and so increase photosynthesis (Lerbs et al. 1984; Schmulling et al. 1997; Husickova 2010).

Pathogen invasion constitutes a key biotic stress to plants. Cytokinins are involved in mediating developmental changes caused by interactions with the pathogen. The interactions between host plants and their pathogens are affected by both changes in endogenous cytokinin content and exogenous applications of cytokinins (Ashby 2000; Bari and Jones 2009). The application of cytokinins to diseased plants has long been known to have positive effects, including both delayed plant senescence and arrest of pathogen development (Husickova 2010).

Dekhuijzen (1976b) reported that callus cells infected with *P. brassicae* did not produce hormones, but pathogen synthesis of cytokinins has been reported (Ludwig-Muller et al. 1996). By contrast, pathogen synthesis of auxins was absent (Ludwig-Muller et al. 1999c) and it appears that the increase in indole-3- acetic acid (IAA) in gall tissue is derived from the host plant (Ludwig-Muller and Schuller 2008). Increased synthesis and turnover of the putative host auxin precursors indole-3-acetaldoxime (IAOx), indole-3-methylglucosinolate (indole GSL) and indole-3-acetonitrile (IAN) have been detected in infected *B. rapa* roots (Searle et al. 1982; Rausch et al. 1983; Butcher et al. 1984).

The role of glucosinolates is intriguing. As result of invasion, indole glucosinolates are found in large quantities in the vacuoles of crucifers. Neuhauser et al. (2011) suggested that the pathogen stimulates infected cells to produce small quantities of glucosinolates in the roots. These act as a semi-sink for sugars, auxins and other plant hormones (Ludwig- Muller et al. 2009; Siemens et al. 2009a,b). Photosynthetic products are transmitted from the leaves to roots, where they accumulate as both starch and hexoses, which are formed instead of fructose biphosphate (Mithen and Magrath 1992; Brodmann et al. 2002). Interestingly, the

glucosinolate content of non-*Brassica* plants inoculated with *P. brassicae* also increased compared with control plants. These included species in the Caricaceae, Resedaceae and Tropaeolaceae and small galls occasionally detected in *Tropaeolum majus* (Ludwig-Muller et al. 1999b) and *Lepidium sativum* (Butcher et al. 1976). It was therefore speculated that these glucosinolates (mainly benzyl glucosinolate) could serve as precursors for phenylacetic acid, a naturally occurring auxin in *T. majus* (Ludwig-Muller and Cohen, 2002) (**Fig. 1.9**).

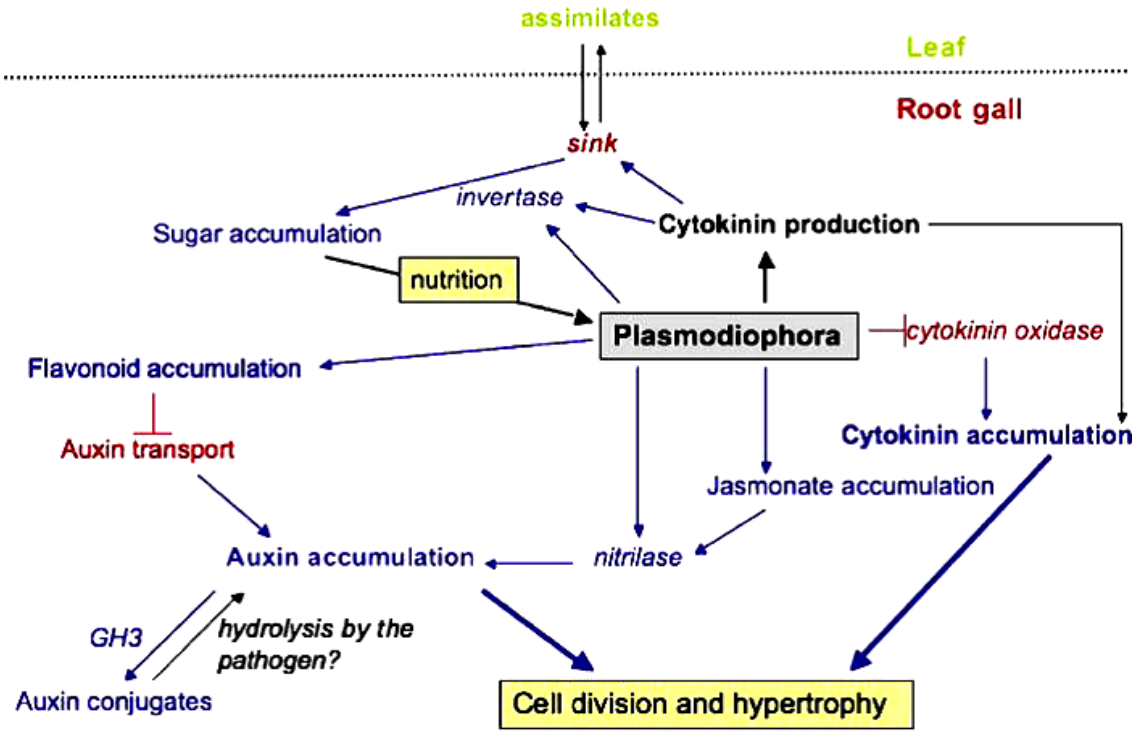

Figure 1.9: A current model detailing hormone homeostasis and metabolism in clubroot of *Arabidopsis thaliana*. Red denotes down-regulation, blue up-regulation of pathways, compounds, enzymes or genes. Black arrows indicate that the pathway is directly influenced *by Plasmodiophora.brassicae*. (from Ludwig-Muller and Schuller, 2008).

In summary, there are many changes that occur during infection. These include up-regulation of some important materials in plant cell walls during the metabolism of polyamines, host (Mithen 1992), defence responses associated with the presence of high concentrations of phytoanticipins, low molecular weight phenolic compounds and phytoalexins, and changes in the amounts and composition of fatty acids (Jubault et al. 2008; Pedras et al. 2008; Ludwig-Muller et al. 2009).

1.3.2 Pathology in susceptible and resistant hosts

Plasmodiophora brassicae infects both susceptible and resistant host plants and this is discussed in more detail in Chapter 6. It was previously thought that only the primary stage of the life-cycle took place in resistant plants (Ludwig-Muller et al. 1997; Donald et al. 2008). Symptoms of disease are only evident as the pathogen completes the secondary stages of its life cycle, resulting in the production of large numbers of resting spores within swollen root cells. It is now clear that resting spores are present, but to a lesser extent, in resistant plants.

Numerous metabolic differences between resistant and susceptible hosts have been recognized. Takahashi et al. (2006) reported that during the infection process the root zone of resistant host plant roots was alkaline whereas that of susceptible roots was not. Moreover, a hypersensitive response (HR) was observed only in the resistant plants. This HR was believed to cause programmed cell death and restrict *P. brassicae* from moving into the cortex. Graveland et al. (1992) reported starch production to be ten times greater in susceptible compared to resistant plants, with the mass and type of starch accumulation being related to the vitality of the host plant. Intact cells of actively growing resistant plants built up crystalline amylose, but infected cells synthesised huge quantities of amorphous amylopectin.

Donald et al. (2008) tracked the life cycle of *P. brassicae* in susceptible and resistant *B. oleracea* (cauliflower). During cortical invasion, the symptoms in infected host plants (both resistant and susceptible) included the presence of ‘vesicles or inclusion bodies, cell wall thickening in association with plasmodesmata and enlarged and/or disorganized host nuclei and cell wall breaks’. The major dissimilarity between the susceptible and resistant host reactions was the absence of degradation of the secondary thickening and cell walls of the xylem in the resistant host.

Differences in glucosinolate metabolism have been reported by Ludwig-Muller et al. (2009). For the first 2 weeks after infection, susceptible and resistant plants had the same level of indole glucosinolates. At 14 and 30 days, aromatic glucosinolates increased in resistant plants whereas at 14 and 20 days indole glucosinolates increased in susceptible plants. As the primary stage of the life cycle takes 14 days and the secondary stage is restricted to susceptible plants, it was proposed that the increase in aromatic glucosinolates instead of indole glucosinolates in the tolerant cultivars after 14 days was a defence response to the pathogen.

1.3.3 Spore survival and dispersal

Historically many researchers have sought to understand the means of transmission of the pathogen and the means by which it can survive for long periods of time (Gaumann, 1950; Kerr, 1980; Davis and Brown 1996) in the hope that his knowledge would reveal a weak link in the disease cycle that could be utilised to control the disease.

P. brassicae can be spread by movement of contaminated soil from an infected site. The movement of contaminated soil has been implicated in the spread of clubroot in many in regions. Cao et al. (2009) documented in their annual surveys in 2005-2008 that the fundamental means of spread of clubroot had been through movement of infested soil on farm machinery, footwear or boots and grazing animals, the use of manure from animals that had eaten infected roots, and compost containing remains of diseased crops (Donald 2006c). Other important means of spread is on the roots of infected transplants, contaminated irrigation water and surface flood water. Dam water, in particular water from dams receiving run-off from infected farms, is also an important means of local spread of *P. brassicae* (Crute et al. 1980); also the dissemination of *P. brassicae* as external seed contamination is possible as suggested by Rennie et al. (2011). In addition, many other factors such as rain, floodwater, sloping land, earthworms, moles, root nematodes and insects can work together or individually to disseminate *P. brassicae* over quite substantial distances (Gleisberg 1922; Chupp 1924; Gibbs 1931; reviewed by Dixon, 2009b). Farm animals and their food supplies sailing with European colonists to the New World and Australasia were probably the means of introducing *P. brassicae* to new virgin territory. In their worldwide survey of physiological races, Toxopeus et al. (1986) indicated a predominance of virulence for *B. napus* in these regions.

Resting spores can survive for long periods in the soil even in the absence of a susceptible host due to the composition of the spore cell wall. Moxham and Buczacki, (1983) stated that the high protein and fat content of the spore wall confers resistance to the spore against digestion by enzymes or other substances naturally present in the soil, and degradation by chemical treatments used to control the pathogen. Spores of *P. brassicae* can remain viable in soil for many years and are stimulated to germinate by chemical compounds in exudates from host plant roots (Suzuki et al 1992). The viability and infection capability of resting spores in soil is affected by many environmental factors, including temperature, soil pH, rainfall,

drainage and water availability (Karling 1968). For instance, when resting spores are at a depth of more than 20 cm they can lose their ability to germinate and cause infection.

1.3.4 Resting spores

1.3.4.1. Shape and size

Resting spores of *P. brassicae* are globose-ovoid and the diameter has been variously reported to be ≤1.6 μm (Woronin, 1878), approximately 3 μm (Buczacki and Cadd, 1976) and 2-3 μm but occasionally 4.6-6.0 μm (Cook and Schwartz, 1930), 3-4 μm (Pinoy, 1907, cited by Buczacki and Cadd ,1976), 1.9-4.3 μm with a mean of 3.3 μm Chupp (1917) and 3.6-4.1 μm with a mean of 3.9 μm (Honig 1931). Jones (1928) observed that the resting spores of *P. brassicae* were almost all spherical, with some being ovoid. Buczacki and Cadd (1976) described the resting spores as subspherical to spherical (**Fig. 1.10**). The surface of each resting spore is covered with spines (Williams and Mcnabola, 1967; Ikegami et al. 1978; Kole and Gielink, 1962) (**Fig. 1.11**). Early although early workers (Woronin, 1878; Cook and Schwartz, 1930) could not see these spines, which were considered to be the result of residual vacuolar material in the host plant (Williams and Mcnabola, 1967) but this now seems unlikely.

Resting spore size is affected by the stage of development. Woronin (1878) and Wellman (1930) reported that measurements taken with an oil-immersion objective averaged 1.7 μm diameter for 'freshly-matured' spores, while in the first stage of germination, swollen by water, resting spores measured more than 2 μm diameter. Naumov (1925) also reported slightly different-sized resting spores produced from different host plants, with spore diameters of 2.9-3.5 μm (mean 3.2 μm); 2.8-3.7 μm (mean 3.3 μm); 2.7-3.3 μm (mean 3.0 μm); 2.5-2.9 μm (mean 3.2 μm); 2.9 – 3.9 μm (mean 3.4 μm) and 2.2-3.0 μm (mean 2.6 μm) from red cabbage, kohlrabi and four sources of swede respectively. This suggests that the diameter is influenced by the environment of the host. Macfarlane (1970) indicated that resting spores of *P. brassicae* were haploid with a single nucleus of 1.5 μm diameter in the centre of the spore.

Figure 1.10: Subspherical to spherical resting spores of *P. brassicae.* Bar=2 µm.

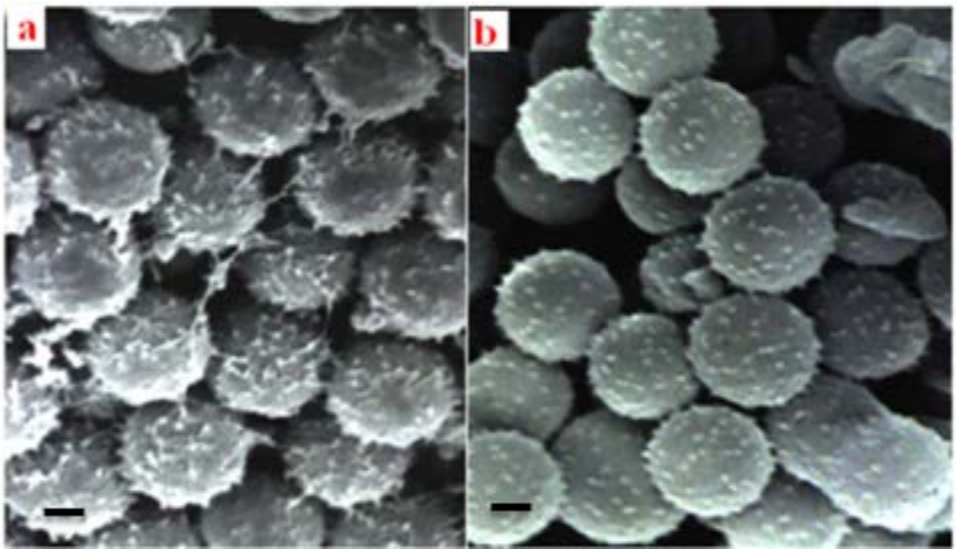

Figure 1.11: Scanning electron micrographs of immature and mature resting spores of *P. brassicae* in turnip. (a) Immature resting spores,(b) Mature resting spores. Scale bar = 1 µm. (from Kageyama and Asano 2009).

1.3.4.2. Spore wall structure and chemical composition

Buczacki and Moxham (1983c) treated resting spores with different substances such as pronase, chitinase, lipase or phospholipases sequentially or following treatment with 5% potassium hydroxide. They reported that some proteins appeared to be bound to the wall by charge or salt linkages, and some were possibly in complexes with lipid material. The resting spore wall had a five-layered structure, where layers 1- 4 were clearly visible, whilst layer 5 (the spore membrane) was closely appressed to the fourth layer (**Fig. 1.12**).

The plant cell wall is the first line of defence and a barrier against potentially harmful biological or chemical agents. Therefore information on the chemical and morphological composition of this wall could be valuable in the development of control measures (Moxham and Buczacki, 1983). The walls of resting spores consisted of 25.5% chitin, 2.5% other carbohydrates, 33.6% protein, less than 17.5% fat and 10.5% residual ash of the total weight (**Table 1.7**) (Moxham and Buczacki, 1983). The lipid and protein content in the wall of spores of *P. brassicae* is very large compared with spores of other microorganisms; this may explain why the spores can survive so long in the soil and are apparently resistant to biocidal agents. Immature spores have a fibrous layer on the spines which is made up of the non-granular constituents of the interspore matrix (**Fig. 1.11**). This may explain why spores clump together in solution. Moxham and Buczacki (1983) showed that there is a small amount of protein that appears to be bound to the wall by charge or salt linkages, some possibly in complexes with lipid material (**Table 1.8**).

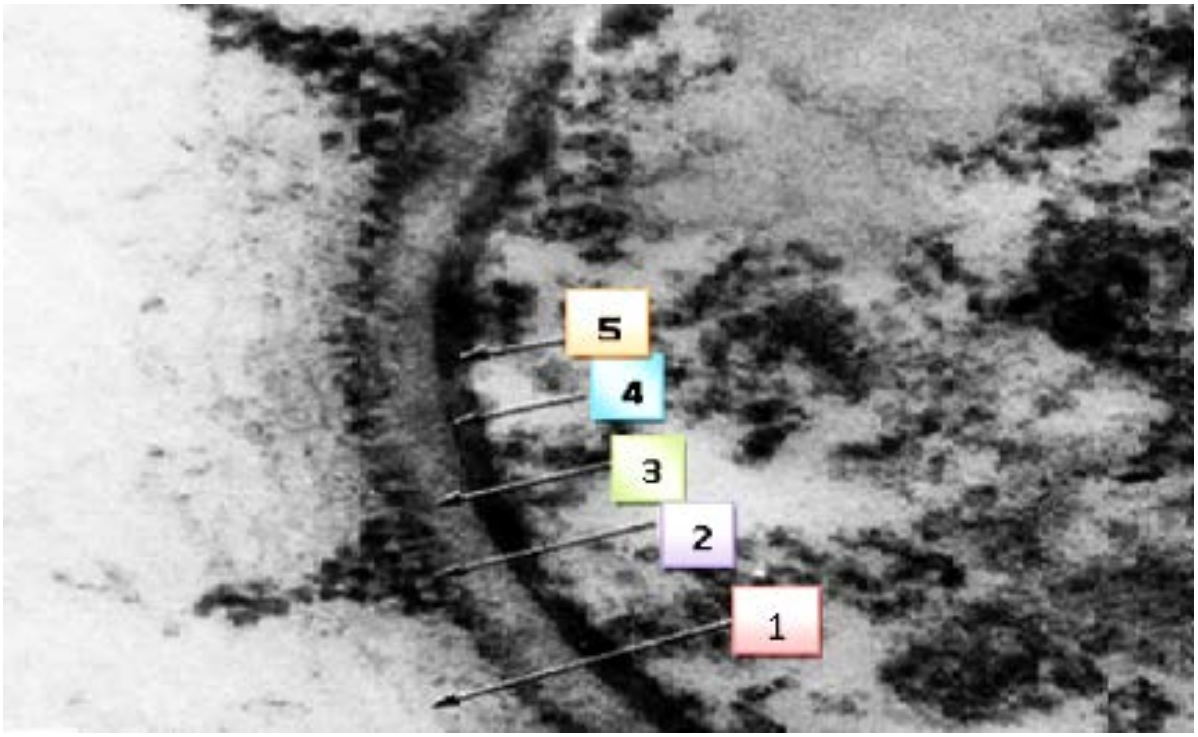

Figure 1.12: Five-layered resting spore wall of *Plasmodiophora brassicae* (Buczacki 1983).

Table 1.7: Composition of *Plasmodiophora brassicae* resting spore walls (Moxham and Buczacki, 1983).

Component	**Wall dry wt (%)**
Chitin	25.1
Other carbohydrates	≥2.5
Protein	33.6
Lipid	≥17.5
Ash residue	10.5
Total	**≥88.3**

Table 1.8: Distribution of *P. brassicae* resting spore wall components (Moxham and Buczacki 1983)

Component	**Analytical method**	**Fraction**	**Wall dry wt (%)**
Reducing sugar	Reducing sugar	80% ethanol	0.3
		1.5 M HCl	14.4
		0.5% oxalic acid	0,7
		6 M HCI	5.2
		Total (sequential hydrolysates)	**20.6**
		Complete hydrolysate	22.4
Non-reducing sugar	Anthrone	Complete hydrolysate	2.5
Hexosamine	Elson & Morgan	Complete hydrolysate	19.4
Glucosamine	Gas chromatography	Complete hydrolysate	22.1
Glucose	Gas chromatography	Complete hydrolysate	0.5
Protein	Lowry	1 M NaOH 25°C	13.7
		1 M NaOH 100ºC	11.2
		1 M acetic acid 100ºC	2.6
		Residue	6.1
		Total	**33.6**
Lipid	Bartnicki-Garcia	Readily extractable	16
	Nickerson/Kessler and Nickerson	Bound	6
		Total	**22**
Nitrogen	Microkjeldahl	Unhydrolysed walls	6.8

1.3.4.3 Germination

Root exudates from susceptible and resistant host plants readily activate resting spores and encourage their germination (Suzuki et al. 1992). Resting spore germination is favoured by moist, acid soil and can occur over a wide temperature range of 12-27°C. Germination takes approximately 18 hours (Wellman 1930). Disease development is favoured by high soil moisture and soil temperatures of 18-25°C. Although clubroot has been found in soils exhibiting a wide pH range from 4.5-8.1, the disease is primarily associated with acid soils, which may promote germination (Colhoun 1953; Zitter 1985).

A single naked zoospore protoplast emerges from a circular pore 1.4 μm in diameter in the wall of each germinating spore (Tanaka et al. 2001). Two unequal anterior flagella appear almost immediately after the cytoplasm emerges from the pore (Ellison 1945). Once liberated, the zoospore protoplasts are slightly ovate and 3-5 μm diameter. These primary zoospores "swim" by means of their flagella to infect susceptible plant root hairs. The number of germinating spores increases over a number of days, indicating that spores do not all germinate at once (Ellison 1945).

1.3.5 Primary zoospore penetration of root hairs

Many researchers have studied the fine structure of *P. brassicae* zoospores, as information on the fine structure of primary zoospores is essential to understand the biology of the primary zoospore and germination mechanisms of the resting sporangium. They have focused predominantly on the germination of primary zoospores and the generation of secondary zoospores (Kole and Gielink, 1962; Dekhuijzen, 1979; Buczacki and Clay, 1984). Tanaka et al. (2001) observed a series of ultrastructural changes in zoospores within resting sporangia of *P. brassicae* and published the first report on primary zoosporogenesis in plasmodiophorids. The processes of primary and secondary zoosporogenesis in *P. brassicae* are fundamentally similar. Both are initiated by the emergence of flagella within resting sporangia and both overall shape and intracellular features of the wall-less zoospores change during development and maturation (Tanaka et al. 2001).

Aist and Williams (1971) reported primary zoospores contacting root hairs many times before becoming attached to one, with the point of attachment being located on the opposite side from flagellar insertion. After attachment, the flagella twist around the zoospore body, until it seems slightly flattened against the host wall. Penetration of the cell wall is by a unique

structure called ‘Rohr und Stachel’ (Agrios 2005). A tubular cavity in the cyst shaped like a bullet (the stachel) is evident within 2 hours of attachment. As a result of pressure on the host cell wall, the rohr is quickly evaginated to form a bulbous adhesorium. The stachel breaks the host wall so quickly that in less than 2 seconds the pathogen is within the root hair. Aist and Williams (1971) reported that the penetration process and the formation of an adhesorium took roughly 1 minute. After penetration, a callose-rich papilla is quickly formed between the host wall and plasma membrane at the penetration point. At this stage, a young amoeba surrounded by a 7-layered envelope and without lipid droplets can be observed in the root hairs.

1.4 Importance of the disease

The family Brassicaeae contains over 330 genera and about 3700 species worldwide (Dixon (2009a). Formerly known as the Cruciferae, because it has four petals held open in the shape of a cross, this family includes many important vegetables and agricultural crops. Many species are infected by a wide range of pathogens such as bacteria, fungi, viruses and nematodes that cause severe destruction to crops, resulting in a loss of yield and revenue to the vegetable industry (**Table 1.9**).

Clubroot has been a significant problem causing economic losses in cruciferous crops for more than a century (Woronin, 1878; Strelkov et al. 2007). Oilseed and vegetable brassica crops are at risk from *P. brassicae*. Clubroot is considered a limiting factor to production in Japan, France, Germany, England, Wales, Scotland, Canada and Australia (Voorrips, 1995; Wallenhammar et al. 2000; Strelkov et al 2007).

Resting spores of *P. brassicae* persist in the soil for many years, making it very difficult to control. Within a single root gall it is possible that the pathogen is heterogeneous in composition (Karling, 1968; Tinggal and Webster and Dixon 1981b; Jones et al. 1982a, b).

1.4.1 Clubroot around the world

Clubroot is one of the oldest plant diseases, recorded as a limiting factor in the production of cole crops such as turnips, which were an important food source for humans and animals in Roman times. One of the first records of clubroot from Italy in the 4th century AD describes ‘paleness’ of the infected plants and ‘spongy roots’ on rape, turnip and radishes grown in soil

fertilized with manure (Watson and Baker 1969). Cattle dung is now believed to be a potential means of spread of *P. brassicae.* (Howard et al. 2010).

Subsequently Diaz de Isla (1539, cited by Howard et al. 2010) described symptoms of clubroot on cabbages in Spain and Fuchs (1542, cited by Watson and Baker 1969) observed a huge spherical gall on the roots of *Brassica oleracea primum.* Howard et al (2010) states that these early reports of clubroot disease support the assumption that clubroot originated in the Mediterranean region not far from the centre of origin of the genus *Brassica*. However, officially Ellis (1750) in England documented the first record of the emergence of clubroot disease; this was followed by a series of reports from across Europe in the next century confirming the appearance and widespread distribution of clubroot disease (Buczacki 1985). The Russian scientist Michael Woronin provided one of the earliest comprehensive descriptions of clubroot disease. From 1860, Russian cabbage crops were heavily infected by *P. brassicae* and there were large losses in the yield in that time (Colhoum 1958; Karling 1968). Woronin in 1878 provided a full description of the disease and identified the causal pathogen, *P. brassicae* Woronin. Later, in 1853, clubroot was recorded for the first time in North America, according to Watson and Baker (1969). It is likely that the use of fodder turnips as food for livestock brought over by early European settlers was an important factor leading to the emergence of clubroot in many countries such as America, Australia and New Zealand (Dixon, 2009a). Clubroot is now found throughout the world wherever *Brassica* crops are grown and is assessed as the major source of disease-induced loss in them (Dixon 2009a).

1.4.2 Clubroot and brassica production in Australia

In Australia, *P. brassicae* causes clubroot disease in vegetable brassicas. It is widespread in vegetable-growing soils and causes significant yield losses of up to 10% annually. In most parts of Australia the pathotypes of *P. brassicae* only cause disease in the warmer months and so crop losses due to clubroot are mainly a summer problem. The exception is Tasmania and some parts of New South Wales, where severe symptoms of disease have been observed during the winter months (Donald and Porter 2003b).

Table 1.9: Important diseases of vegetable brassicas (e.g. broccoli, cabbage, cauliflower and Chinese cabbage and Brussels sprout).

Type of pathogen	Disease	Pathogen	Reference
Bacteria	Black rot and leaf scald	*Xanthomonas campestris pv. campestris*	Denis et al. 2010; Williams 1980.
	Head rot	Several species of bacteria, including *Erwinia* and *Pseudomonas*	Lancaster 2006; Denis et al. 2010.
	Soft rot	*Erwinia carotovora*	
	Peppery leaf spot (Bacterial leaf spot)	*Pseudomonas syringae* pv. *maculicola*	
	Zonate leaf spot	*Pseudomonas cichorii*	
Fungi	Leaf spot / target spot (*Alternaria spot)*	*Alternaria brassicae, A. brassicicola*	Denis et al. 2010.
	Black leg	*Leptosphaeria maculans*	Sharf 1968; Denis et al. 2010.
	Black root of radish	*Aphanomyces raphani*	Denis et al. 2010; Wenham 1960.
	Clubroot	*P. brassicae*	Denis et al. 2010; Woronin 1878.
	Downy mildew	*Halyoperonospora parasitica, Peronospora parasitica*	Choi et al. 2003; Denis et al. 2010.
	Light legs spot	*Cylindrosporium concentricum*	Denis et al. 2010; Gilles, 2000.
	Ring spot	*Mycosphaerella brassicicola*	Lancaster 2006, Denis et al. 2010.
	Stem canker	*Several pathogens, e.g. Phoma, Fusarium,*	Denis et al. 2010.
	White blister (White rust)	*Albugo candida*	Ferreira and Boley, 1991, Denis et al. 2010.
	Yellows (*Fusarium* wilt)	*Fusarium oxysporum* f.sp. *conglutinans*	Denis et al. 2010, Khangura 2009.
	Powdery mildew	*Erysiphe cruciferarum*	Lancaster 2006, Sherf and MacNab 1986.
	Damping off	*Fusarium* species or *Pythium* species	Lancaster 2006, Matta 1965.
	Grey mould	*Botrytis cinerea*	Bishop and Reagan, 1998.
	White mould	*Sclerotinia* species	Lancaster 2006, Kohn 1979.
	Wire stem	*Rhizoctonia solani*	Hansen 2009, Lancaster 2006.
	White leaf spot	*Pseudocercosporella capsellae*	Lancaster 2006.
Viruses	Turnip mosaic virus	TuMV (Potyvirus)	Denis et al. 2010.
	Cauliflower mosaic virus	CaMV (Caulimovirus)	
	Beet western yellows virus	BWYV (Polerovirus)	
Nematodes	Root-knot nematode	*Meloidogyne* spp.	Lancaster 2006.
	Cabbage and cauliflower cyst nematodes	*Heterodera cruciferae, H. schachtii*	Aydinli and Sevilhan 2012.

The Australian vegetable brassica industry produces $134 million worth of crops annually (Donald, 2000). All of these crops are susceptible to *P. brassicae*. A survey of Victorian brassica growers found that over 70% of properties were affected by clubroot. Crop losses of up to 25 hectares/property were reported and total national crop loss was estimated at at 5-10% of *Brassica* production or approximately AUD$10 m annually (Donald 2003a).

Clubroot is now endemic in most of the major production regions of Victoria, New South Wales and Tasmania (Donald 2003a). Outbreaks were first reported in Manjimup (Western Australia) in 1993, Stanthorpe (Queensland) in 1997 and Gatton (Queensland) in 2001. With the exception of Gatton, which has a naturally high soil pH and where a summer break from *Brassica* crops is routinely practised to manage insect pests, clubroot is now widespread in these areas and is a problem in every state of Australia where vegetable brassicas are grown in Australia (Donald, 2003a; Donald and Porter 2003b).

1.5. Effect of environmental conditions

P. brassicae is an obligate pathogen and therefore cannot be cultured. The resting spores of the pathogen, which are its long-term survival structures and the means of primary infection, can be stored at -20ºC for many years with minimal loss of viability, though no more than 3 years is recommended (Donald, 2003a and Donald and Porter 2003b). Inoculum can be prepared from symptomatic roots by mixing with water in an electric homogenizer. The resulting slurry should then be filtered and cleaned by centrifugation as described by Williams (1966). The spores can be quantified using a haemocytometer or flow cytometer and the concentration of the final solution varied as required. Resting spores can be used to inoculate host plants by application directly to seedling roots or mixing with compost or soil into which seed or seedlings are placed. Using these methods, the inoculum densities required to induce 50% disease incidence varied from 10^3-10^6 resting spores mL^{-1} (Dixon 1976a; Voorrips 1996a,b). Similarly, Wallenhammar (1996) stated that symptom development requires a concentration of 1000 spores g^{-1} soil.

Inoculated test plants should be maintained at a basal temperature of 20–25ºC for up to 6-7 weeks, by which time galling symptoms will be apparent. On the microgeographic scale there is substantial physiological variation, while on the world scale it reflects the dominant species of *Brassica* most commonly cultivated in a particular geographic area (Toxopeus et al. 1986).

The ability of the pathogen to attack its hosts affects the severity of the disease; this depends on the abundance and virulence of the pathogen and also the susceptibility of the host. Environmental factors affect the development and productivity of crops and also affect the severity of the disease. Since *P. brassicae* is a soil-borne pathogen that affects the root systems of the infected plants, soil moisture, temperature, pH and mineral composition all have an impact on clubroot severity.

1.5.1 Temperature

Temperature is regarded as one of the most influential environmental factors to affect the development of pathogens and the diseases caused (Colhoun 1973). The most important problem in conducting these experiments is maintaining precise control of temperature during the studies. In general, the greatest infection arises with temperatures in the low twenties and temperatures of <15°C or >30°C suppress disease.

Early study conducted in a glasshouse by Monteith (1924) showed that clubroot symptoms developed on cabbage at soil temperatures of 15-30°C, while they did not if the temperature was less than 12°C. A subsequent study showed that the temperature for maximum resting spore germination was 25ºC, with 18ºC and 35ºC being the lower and higher limits respectively (Wellman 1930). Buczacki et al. (1978) reported 100% infection with a minimum mean temperature of 19.5ºC during the second week after planting.

McDonald and Westerveld (2008) similarly showed that soil temperatures of less than 12ºC in the 10 days prior to harvest were correlated with low incidence and severity of clubroot at harvest of Chinese cabbage and ‘Shanghai’ pak choy. Gossen et al. (2012b) similarly demonstrated that temperatures of 20–26ºC caused the greatest amount of clubbing in ‘Shanghai’ pak choy and canola, but 17ºC or less totally suppressed clubbing and 30ºC or above decreased it. Suppressive temperatures such as <17°C affected the disease similarly across each stage of plant development; in the first 3 weeks after sowing it inhibited root hair infection and symptom development and in later weeks (4–6 weeks) it inhibited the incidence and severity of clubbing.

Both air and soil temperatures affected symptom development and severity at all stages of disease progression (Thomas 2013**).** Sharma et al. (2011a) showed that root hair infection occurred fastest at 25ºC and slowest at 10ºC. In ‘Shanghai’ pak choy grown at 25ºC, root hair infection occurred at 2 dai and taproot swelling was observed at 10 dai. Both higher and

lower temperatures correlated with slower infection. While root hair infection first appeared at 4 dai in plants grown at 15, 20 and 30ºC, it appeared later, at 6 dai, at 10ºC. Cortical infection also occurred earliest and to the greatest extent at 25°C, whereas the lower limit for cortical infection and gall development was 10-15°C (Sharma et al. 2011b). Lastly, Dixon (2009b) stated that a soil temperature of 24ºC with pH 6.0–6.7 provided the most favourable conditions for the germination of resting spores, while temperatures of 45ºC or greater were lethal to the pathogen.

Thuma et al. (1983) noted that the potential to predict clubroot severity in the field depended on accumulated 'degree days' based on soil temperature, which were highly associated with clubroot severity on radish. There was also a high correlation between air temperature and severity; however, the relationship was not as strong as that with soil temperature.

Monteith (1924) found that interaction between temperature, soil type and moisture content could affect clubroot severity; for instance, some clubbing occurred at a soil temperature of 9°C on cabbage grown in sandy loam soil with high organic content and maintained at 90% soil moisture, but not in a clay loam soil maintained at 75% soil moisture; also, there was severe galling at a soil temperature of 20°C and soil moisture of 75% of water-holding capacity but this decreased with increasing temperature. Colhoun (1952) also demonstrated an interaction between soil pH and temperature; in alkaline soil with a pH of 7.8, the greatest clubroot incidence occurred with a mean air temperature of $\geqslant$23ºC. Similarly, there is also an interaction between temperature and pH; clubroot severity was >20 DSI in canola plants at 10 or 15°C with pH of 6.0-8.0 (Kasinathan 2012).

1.5.2 Soil pH

Soil pH affects the incidence and severity of clubroot disease and liming (adding $Ca(OH)_2$) is a common preventative treatment that raises soil pH. Rastas et al. (2012) found in Finland from a field survey that clubroot was more severe and occurred more frequently in soils with pH <6.5 than in soils with pH >6.5. Similarly, infection and subsequent clubroot symptom development were mostly greater at pH 5.4-7.1 than at pH 7.3-8.0 (Colhoun 1953; Myers and Campbell 1985), while resting spore germination was slower in limed soils with pH 8.0 than in acidic soils of pH 5.8 (Macfarlane 1952).

Root exudates can increase resting spore germination and affect pH. Takahashi et al. (2006) showed that root exudates from resistant plants raised the pH of their surroundings. Clubroot-

resistant roots cultured in Murashige and Skoog agar medium and exposed to resting spores increased the alkalinity of their culture medium from pH 5.2-5.6 in 3 days but the pH did not change for cultures of susceptible roots. More recently, Rashid et al. (2013) investigated the effects of root exudates and pH on resting spore germination and infection of canola (*B. napus*) and found that root exudates from two host species for *P. brassicae* (canola and the highly susceptible Chinese cabbage cv. Granaat) increased the proportion of resting spores that germinated compared with mineral nutrient solution alone or with sterile distilled water. Surprisingly, root exudates from the non-host plant perennial ryegrass (*Lolium perenne*) produced greater spore germination than root exudates from these host plants. Resting spore germination was greater at pH 5.0-7.0 than at pH >7.0 in both the presence and absence of root exudates. Root hair infection was also greater at pH 6.0-7.0 than at any lower or higher pH in mineral nutrient solution, though it was less at pH <6.5 than pH>6.5 without mineral nutrient solution. The ions in the mineral nutrient solution, particularly Ca^{2+}, may have mitigated the pH effect.

Calcium interacts with pH to suppress clubroot but may have an independent role. Calcium treatments at pH 6.2 produced as much inhibition of clubroot symptoms at low inoculum levels as those at pH 7.2 at high inoculum levels (Webster and Dixon, 1991a). Calcium can also play a role in resistant plants to mediate induction of phenylalanine ammonia-lyase (PAL) activity as a defence response to contact by *P. brassicae*. PAL, in turn, is necessary for the expression of clubroot resistance in turnip (Takahashi et al. 2002). Dixon (2009b) suggested that hydrogen cations might compete with or act against calcium in the host plant tissues and so low pH increased symptoms; by contrast, alkaline pH increases calcium absorption in roots (Kasinathan 2012; Kasinathan et al. 2010).

There is also an interaction between pH and temperature; the greatest clubroot severity developed in canola at pH 6.0 and 25ºC, while disease intensity dropped but disease still developed (to 40 DSI) at the optimum temperature at pH 8.

1.5.3 Soil moisture

There is an inverse relationship between clubroot severity and soil moisture (Monteith 1924). Soil moisture affects the motility of zoospores in soil, consequently affecting their ability to migrate to roots (Colhoun 1973); soil moisture of 70% was the most favourable for resting spore germination and infection of roots. Soil that is too dry or too wet suppresses clubroot.

Clubroot develops in soil with soil moisture of 60-100%, but does not develop in soil with ≤45%; also clubroot incidence was greater (100%) on turnip rape irrigated daily to maximum holding capacity than in plants watered only when they showed symptoms of wilting (60%) (Thomas 2013). Rastas et al. (2012) also showed that daily irrigation resulted in poor yield (500 kg ha^{-1}) compared with the wilting-only treatment (1,500 kg ha^{-1}). Rainy days at 2-3 weeks after sowing were positively correlated with clubroot severity, as was total rainfall with incidence and severity in vegetable crops on muck soils (Thuma et al. 1983; Gossen et al. 2012a). Likewise, dry soils with moisture levels of ⩽30 % delayed resting spore germination (Macfarlane 1952).

1.5.4 Spore load

The resting spore density in the soil can be high following an infected crop, as infected plant roots rot and release spores. Susceptible volunteer canola growing in the same field as resistant canola developed clubroot symptoms and consequently increased the resting spore density in soil. The presence of large quantities of the pathogen increases the chance of infection; the number of root hair infections increased with the concentration of resting spores in the soil for crops such as canola, cabbage, kale, cauliflower, Brussels sprouts, turnip, radish and garden cress (Macfarlane, 1952; Hwang et al. 2011a,b). Resting spore levels generally need to be ⩾1000 spores g^{-1} of dry soil for gall development (Dixon 1976a; Voorrips 1996a,b; Wallenhammar 1996; Donald and Porter 2009; Faggian and Strelkov 2009). However, clubroot developed on napa cabbage cv. Shin-Azuma when resting spore concentrations were only 10 spores g^{-1} soil (Murakami et al. 2002). Like other plant pathogens, increasing dose increases disease. Increasing inoculum concentration from 10^5 to 10^8 resting spores cm^{-3} increased clubroot severity in canola (Hwang et al. 2011c) and the amount of seedlings, plant height and seed yield per pot of a susceptible canola cultivar reduced exponentially with increasing inoculum (Hwang et al. 2011b). Also, increasing inoculum concentrations, from 10^3 to 10^7 resting spores g^{-1} of dry soil, increased clubroot severity and decreased foliar weight in napa cabbage (Hildebrand and McRae, 1998).

1.5.5 Light intensity

Dixon (2009b) noted that ‘the light intensity received by the host affects the severity of galls’. Increasing the light energy accumulated by the host may increase photosynthate availability. Infected cells in susceptible plants have large quantities of starch grains around the plasmodia

while resistant plants do not (Keen and Williams 1969; Graveland et al. 1992; Morgner 1995, cited in Kobelt et al. 2000; Tanaka et al. 2006), supporting the suggestion that photosynthate supply is vital for energy by the pathogen. Light may also have other effects. Buczacki et al. (1978) suggested that increasing light energy could increase the accumulation of host glucobrassicin, a precursor of indoleacetonitrile, high levels of which are associated with gall development. Dixon (2009b) also suggested that light could be considered as inhibitory to resting spore germination.

1.6 Detection of the pathogen

The ability to detect and quantify *P. brassicae* resting spores in the soil and therefore predict potential yield loss is an important tool to assist *Brassica* growers in the selection of crop management practices. Several techniques have been developed to detect *P. brassicae* in plant tissue and field soils and to study the genetic variation within and between field isolates. These range from baiting techniques requiring plants to be grown under controlled conditions to molecular techniques based on DNA.

1.6.1 Bioassay tests (baiting)

Plant baiting has been used for many years as an indicator of the presence of an infective dose of inoculum (Colhoum, 1957). This technique, based on visual observation of symptoms, was widely used for many years for detection of resting spores of *P. brassicae* present in field soil. Samuel and Garrett (1945) developed one of the earliest fast plant baiting techniques for *P. brassicae* by using a simple acetocarmine stain and microscopic observation to find zoosporangia in the root hairs. The reliability of this method is influenced by exogenous factors, including nutrient availability and environmental factors. It is also time-consuming to perform and has since been replaced by faster and more reliable molecular techniques.

Plant baiting techniques are also routinely used to study variation in pathogen populations. Early researchers observed significant differences in the degree of infection and clubbing that occurred on different *Brassica* crops, concluding that *P. brassicae* contains multiple physiological pathotypes with varying degrees of host specificity and virulence (Karling, 1968; Buczacki et al. 1975). As a result of the wide range of *Brassica* crops that can be attacked by *P. brasssicae*, researchers developed many different kinds of differential host plant sets to study variation in pathogen populations (Williams 1966; Some et al. 1996; Buczacki et al. 1975; Donald et al. 2006a; Scholze et al. 2002; Aydin et al. 2010). Most of

these classified the pathogen into simple groups based on the degree of clubbing (e.g. Williams 1966). The use of a number of dissimilar differential hosts by different research groups made it difficult to compare the results from these studies (Chambers 1977). Buczacki et al. (1975) attempted to rationalise experimental procedures and developed an internationally accepted system called the European Clubroot Differential set (ECD) to characterize populations of *P. brassicae.* In brief, the European Clubroot Differential set (ECD) consists of a collection of *Brassica* genotypes with different numbers and types of resistance genes to differentiate between isolates based on the reactions between the pathogen and the host plants as well on their virulence phenotype. The ECD set of host plants is now widely accepted and used. It consists of 15 *Brassica* plants, five differential hosts in each of three species groups: *Brassica campestris, B. napus* and *B. oleracea* (**Fig. 1.13**). Each host within a species group is ascribed a denary number (1, 2, 4, 8 or 16). The denary numbers of hosts in each species group producing a susceptible reaction are added together. The three numbers, one for each species group, provide a unique code to identify the pathogenicity of the isolate (Buczacki et al. 1975; Kuginuki et al.1999).

Plant baiting techniques, including the European Clubroot Differential set, are labour-intensive and time-consuming to conduct and require trained personnel, particularly if root hair assessment is required, and a large area of glasshouse space (Melville and Hawken 1967; Wakeham and White 1996; Manzanares et al. 2000a). For practical and economic reasons, plant baiting techniques cannot form the basis of a routine diagnostic method for large numbers of samples. The outcome, based upon visual assessment of disease symptoms, is influenced by environmental factors. Often, reactions on these hosts are indeterminate and the symptom scores are variable for some field isolates. Repeated tests using the same pathogen collections sometimes do not produce the same result, probably due to multiple pathotypes within the same collection of root galls (Kalpana et al. 2012) and the heterogeneity of field populations (Fahling et al. 2003). Moreover, pathogenic genotypes might be masked (or influenced) by non-pathogenic genotypes in a root gall. In addition, many of the test species are open-pollinated and so themselves have a degree of heterogeneity, affecting results with successive batches of seed. Reliable classification using these methods is therefore difficult and a more reliable screening method is required through the use of genetically uniform differential hosts (Kuginuki et al. 1999).

1.6.2 Molecular techniques

Faggian and Strelkov (2009) emphasised that bait plants are the most reliable diagnostic method for evaluating soils for *P. brassicae* and they are still intensely relied upon today despite the availability of more modern molecular techniques. In general, baiting methods are still used to confirm the presence of the disease in the field and as the only means of validating newly developed diagnostic procedures (Wallenhammar 1996; Cao et al. 2007).
Molecular techniques can, however, provide precise, reliable and reproducible results rapidly, facilitating early disease management decisions (Faggian et al. 1999). Biochemical, immunological and nucleic acid-based assays have been preferred, because of their distinct advantages over conventional methods. Molecular techniques have been very useful in the identification of obligate pathogens causing diseases such as clubroot, and also for fungi that grow very slowly in culture media, taking several weeks to produce spore forms that can be used for identification. For example, detection of soil-borne pathogens has posed intractable problems because of their irregular distribution and the presence of other microorganisms in the soil. Molecular methods based on the polymerase chain reaction (PCR) using primers targeted to the ITS region of *P. brassicae* ribosomal RNA region have been effective for the detection of *P. brassicae* in soil (Faggian et al.1999; Ito et al. 1999b; Wallenhammar and Arwidsson 2001; Cao et al. 2007).

PCR-based diagnostics are very sensitive, are commonly used in the laboratory for specific detection of *P. brassicae* DNA, and correlate quite well with disease severity. Faggian et al. (1999) and Ito et al. (1999b) reported significant improvement in the sensitivity of detection using nested PCR. Using primers PBTZS-1 and PBTZS-2 to amplify a 1457 bp fragment from *P. brassicae* DNA and nested primers PBTZ-3 and PBTZ-4 to amplify a 398 bp fragment internal of the 1457 bp fragment in a single-tube nested PCR (STN-PCR) reaction, Ito et al. (1999b) could detect even a single resting spore present in 1 g of soil. There are, however, some difficulties in using DNA-based methods with *P. brassicae*.

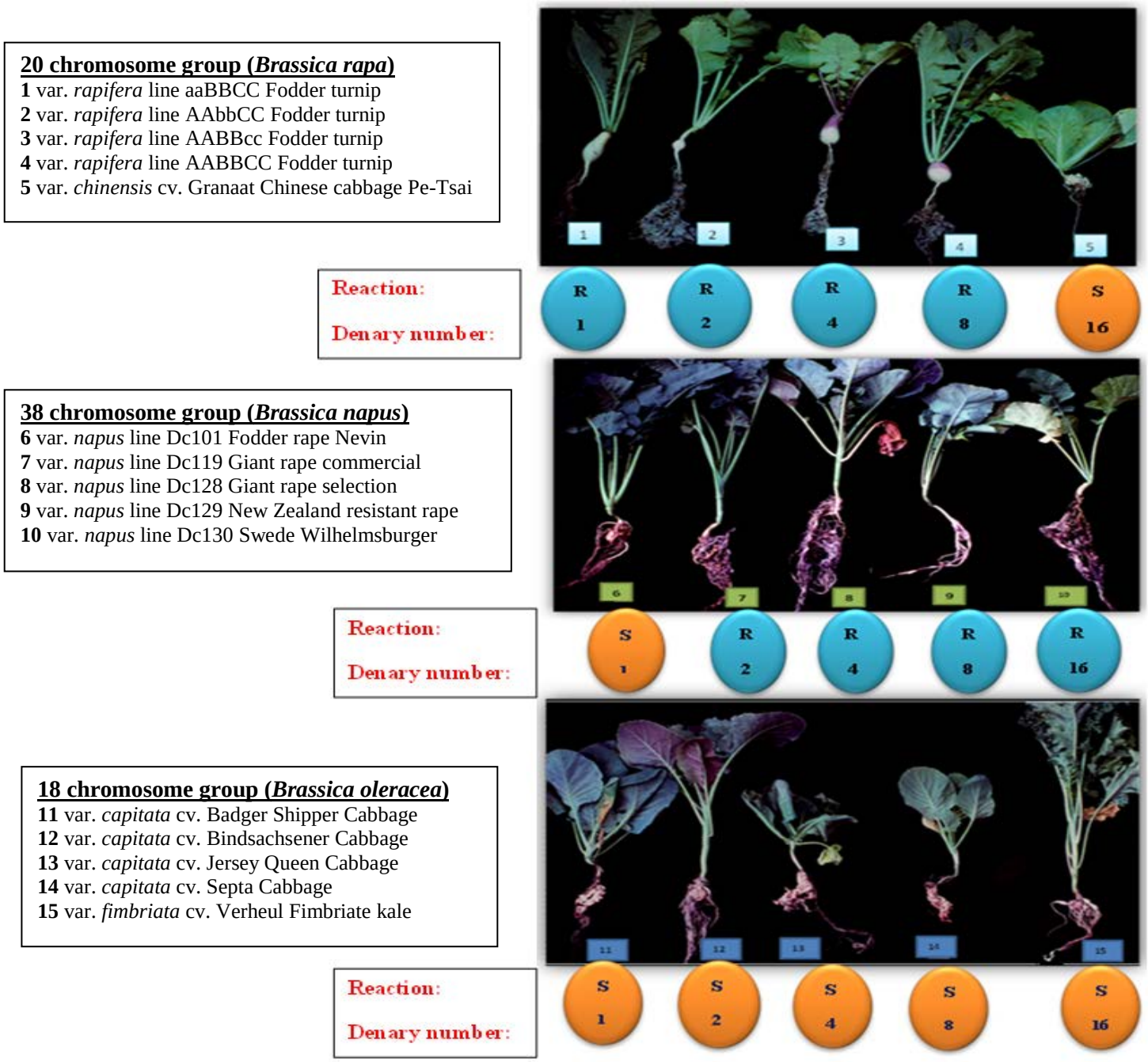

Figure 1.13: Reactions (R=resistant, S=susceptible) of the European Clubroot Differential series of *Brassica* hosts inoculated with *P. brassicae.* ECD code 16/1/31 assigned to this isolate is the sum of the denary numbers of susceptible hosts in each species group (circled).

Siemens et al. (2009a) stated that one of the most important difficulties when working with *P. brassicae* is obtaining pure genetic material (DNA) free from contamination with host plant materials or with any other organism. As *P. brassicae* is an obligate biotroph, genetic materials must be extracted from resting spores of the pathogen obtained from root galls. While these preparations are highly enriched with *P. brassicae* nucleic acids, non-specific microbial contamination is present and poor yields of RNA are obtained from *P. brassicae* spores. This has so far prevented EST (expressed sequence tag) sequencing from this material. Bulman et al. (2006) have located genes of the pathogen in roots of the model organism *A. thaliana* by inhibiting hybridisation between RNA from *P. brassicae*-infected and uninfected plant tissue as well as screening full-length cDNA clones from the infected tissue using an oligo-capping procedure. The result showed nine new genes after genomic DNA sequences flanking 18 of the *P. brassicae* sequences were searched through BLASTX and confirmed by RACE amplification.

A second difficulty is that populations of *P. brassicae* exist as mixtures of pathotypes in the soil and in single root galls (Jones et al. 1982a,b; Fahling et al. 2003; Siemens 2009a) and so DNA extracted from galls is necessarily a mixture of genotypes. To simplify working with such genetically heterogenous materials, single spore isolate (SSI) lines have been generated, but the process of generating SSI lines is time-consuming and has a low success rate. Alternatively, since most *P. brassicae* samples used for experimentation are likely to be heterogeneous mixtures of genotypes, the use of methods such as denaturing gradient gel electrophoresis (DGGE) that can differentiate these would be beneficial.

With the advent of real-time PCR instruments, quantitative PCR is now used widely for the accurate detection and high throughput quantification of fungal pathogens from various samples such as host tissue, soil and water (Schena et al. 2004) and Faggian (2002) used a qPCR assay for the quantification of *P. brassicae* spores in infested soil. This method has much promise but uses expensive equipment and reagents, much more so than normal PCR. According to the recent review by Faggian and Strelkov (2009), the high cost of processing samples by PCR might not always be practical for the farmer. The development of an inexpensive diagnostic kit for the assessment of clubroot inoculum in the field is a priority and immunological assays such as dot-blots may be the future (Faggian and Strelkov 2009).

1.7 Genetic diversity

Many studies have been conducted to determine variation in the pathogencity of *P. brassicae* (Ayers 1957; Williams 1966; Johnston 1968; Buczacki et al. 1975; Tanaka et al. 1997). By studying the pathogenicity of isolates to cruciferous species and varieties, these authors confirmed the physiological diversity of races in the pathogen population (Cho et al. 2003). Dixon (1980) reported the existence of physiological races in the population of *P. brassicae* in different host plants (e.g. in the universally susceptible *B. campestris* ssp. *pekinensis*) and suggested that hosts with different resistance at the same site may select out *P. brassicae* with different patterns of virulence in the field.

1.7.1 Detection of variation

Many studies have evaluated genetic variation between and within field-derived SSIs using molecular markers (this is discussed further in Chapter 5). These markers can distinguish many more genotypes than differential hosts, and showed at least a comparable level of genetic diversity. The variation in distribution of virulence is at very small scale, down to SSIs from a single gall field isolate, indicating that some field isolates are very complex composite populations of a mix of genotypes. Furthermore, there was pathotype inhibition during pathotyping of the field isolates and selection for specific pathotypes during the SSI extraction process in some cases (Crute et al. 1980; Linnasalmi and Toiviainen, 1991; Buhariwalla et al. 1995a; Voorrips 1995; Moller and Harling 1996; Kuginuki et al. 1999; Manzanares et al. 2001).

Suitable methods to assess the genetic diversity within and between populations and field isolates are now available (Buhariwalla et al. 1995a). The genetic diversity and pathogenicity within *P. brassicae* has been studied by a number of researchers in the last 20 years (Buhariwalla et al. 1995a; Moller and Harling 1996; Yano et al. 1997; Manzanares et al. 2001; Fahling et al. 2003; Osaki et al. 2008a). Some et al. (1996) showed variation in virulence amongst 20 field collections of *P. brassicae* from France, confirming the genetic heterogeneity of field populations of *P. brassicae*. This is influenced by the genetics of the host on which *P. brassicae* is assayed. Hansen (1989) reported genetic additive variation as well as non-additive variation, mainly caused by dominance, in a study of genetic variation and inheritance of tolerance to clubroot. Moreover, all characters under investigation (e.g. stem length and storage quality) showed continuous variation.

Williams (1966) and Buczacki et al. (1975) described variation in pathogenicity between populations based on the reaction of a set of differential hosts following inoculation. Clubroot in the weed *Cardamine flexuosa* is widely distributed in most regions of Japan. Tanaka et al. (1993, 2006) showed that populations of *P. brassicae* from this weed were only slightly pathogenic on cruciferous crops, prompting questions about how the unique populations arose and about the origin and evolution of *P. brassicae* in Japan.

Both microsatellite-targeted and randomly amplified polymorphic DNA (RAPD) primers have been used to study genotype variation in *P. brassicae*. Buhariwalla et al. (1995a) provided one of the first descriptions of the use of clubroot-specific SG primers such as HKB17/9, HKB17/33, HKB23/52, and RAPD primers such as OPA-01,OPA-14 and OPA-20 as effective tools for studying the biology and genetic variation of *P. brassicae*. They noted the difficulty of interpreting the amplification of the pathogen with RAPD primers because of the potential contamination of resting spores of *P. brassicae* by host plant or microorganism DNA during extraction. Crute et al. (1983) and Manzanares et al. (2001) found no clear relationship between molecular analyses and pathogenicity tests, ascribed this to contamination by foreign DNA and proposed that a main focus should be obtaining pure DNA for the pathogen. By contrast, Moller and Harling (1996) and Manzanares et al. (2001) emphasised that amplification of *P. brassicae* with arbitrary primers has proved to be a successful means of characterizing genetic diversity. RAPD with Operon primers and standardized conditions of *P. brassicae* DNA extraction produced simple amplification patterns that could be clearly interpreted.

Osaki et al. (2008a) studied genetic diversity within 17 populations of *P.brassicae* in Japan and showed a comparatively high level of diversity, with some populations considered extremely heterogeneous. They proposed that the populations were probably genetically polyphyletic and that divergence could have occurred during parallel evolution. No evidence was found for any pattern relating the geographical distribution to the genotypes.

RAPD analysis has also been used to find molecular markers of resistance in plant hosts. A study of cultivars of Chinese cabbage in Japan found that some cultivars, e.g. ‘Utage’, had at least one specific resistance gene not found in other cultivars (Kuginuki et al. 1999). Yano et al. (1997) demonstrated that RAPD analysis could differentiate some of Williams’ race populations, but were unable to differentiate virulent populations bred in Japan.

1.7.2 Virulence genes

Attempting to breed resistance into *Brassica* cultivars requires adequate testing against a wide range of phenotypes and genotypes of *P. brassicae*. Pathotype testing, while essential, can be daunting in its scale and variable in its results. Crute et al. (1980), Scholze et al. (2002) and Donald et al. (2006b) suggested that different ECD codes resulting from the same field population might be due to the presence of a mixed population of resting spores being present in the root galls used to prepare the spore suspension to inoculate the host plants. Additionally, Donald et al. (2006b) suggested that different environmental conditions (soil moisture, soil pH, temperature and light) could play an important role in determining the differences in virulence within the population.

Genotype testing as the first step is a more reasonable proposition to select the dominant genotypes to be used for pathotype testing, but populations of field isolates of *P. brassicae* consist of mixture of genotypes, and may carry different avirulence or virulence genes. Varying levels of virulence can be observed within a virulent pathogen population (Cao et al. 2009). This high level of variation is desirable for pathogen survival. Cho et al. (2003), Donald et al. (2006b) and Hirai (2006) demonstrated genetic variation in field populations of *P. brassicae.* This variation may enable the pathogen to overcome host resistance and is a huge challenge for the development of clubroot-resistant varieties. Some et al. (1996) and Manzanares et al. (2001), in their studies of the variation in virulence amongst 20 field collections of *P. brassicae* from France, confirmed that the SSIs and their parental field populations were highly heterogeneous for both virulence and DNA polymorphism patterns. Manzanares et al. (2001) emphasised the difficulty of choosing breeding tactics to develop durable clubroot resistance because of the ample genetic variation in *P. brassicae* populations.

1.8 Clubroot disease control strategies

Once crops are infected, and the pathogen has established in the soil, it is very difficult to eradicate *P. brassicae*. Affected areas cannot be used for the cultivation of crucifers until costly soil sterilisation has been carried out. No completely effective fungicides are presently available for controlling clubroot and disease control strategies are limited to sanitation and the use of resistant cultivars, which are not available for many crucifers.

An effective disease management option must be economical; that is, the value of the crop saved must exceed the cost of control. For this reason, assessments of disease incidence,

disease severity, and potential crop loss are key factors when considering control strategies. The strategies implemented also differ depending upon the value of the crop. For example, expensive fungicide treatments may be considered for high value horticultural crops but these would be uneconomical for broadacre oilseed crops.

Clubroot is a difficult disease to control. This is due in part to resilient resting spores which confer the ability to remain infectious in the soil for many years even in the absence of a susceptible host. The most effective means to avoid significant crop loss is to cultivate crops in soil that is completely free of the pathogen or to avoid planting cruciferous crops in *P. brassicae*-infested soil (Cao et al. 2007; Faggian et al. 1999). In most intensive *Brassica* production systems this is not a feasible option and crop rotation, increasing the soil pH and use of resistant varieties remain the common methods currently used (Ludwig-Muller et al. 1999b).

1.8.1 Host resistance

Disease resistance in *Brassica* varieties is very important to growers; in particular resistance to clubroot is high priority. In general, disease-resistant plants are an obvious and effective control measure because resistance can be both complete and long lasting. A plant can express resistance through the action of a single gene that confers immunity or through multiple genes that result in a broad resistance to many pathogens. Single-gene resistance, called *vertical resistance,* limits both the initial level of infection and the production of inoculum. This sort of resistance can be overcome, however, by new strains of the target pathogen. Multiple-gene resistance, called horizontal resistance, allows some disease to develop but limits it to a tolerable level. The status of the development of commercial disease-resistant varieties has been reviewed by Diederichsen et al. (2009). Considerable research effort has been directed towards the development of disease-resistant varieties, but the genetic diversity of the pathogen makes it difficult to control.

Crete and Chiang (1980) stressed the need to search for resistant varieties in Brassicaceae after they screened 109 *Brassica* crops from different countries including China, India, Sweden, Denmark, Poland, Australia, Egypt, Israel, USA, Taiwan, USSR, Turkey, Netherlands, Denmark and Kenya, in order to assess the degree of resistance to clubroot. Most of the lines were infected by *P. brassicae* race 6, ECD code number 16/02/30. Of the

107 infected crops, reduced infection (approximately 50%) was recorded in only two *B. rapa* lines.

Clubroot-resistant cultivars of Chinese cabbage have been used in Australia but breakdown of resistance has been reported for some resistant varieties. Clubroot resistant varieties of *B. oleracea* have become available commercially in Australia (Diederichsen et al. 2006; Donald and Porter 2009); however, use of these varieties has been limited because the produce does not suit market requirements or the varieties are not adapted to local environments (Donald 2003a). Transgenic resistant or tolerant mutants have been reported but public concern over the safety of transgenic plants has prevented the commercial use of these crops (Fuchs and Sacristan 1996; Narisawa et al. 2000; Siemens et al. 2002; Siemens et al. 2006; Alix et al. 2007).

Jubault et al. (2013) stated that, to date, most of the studies of the molecular basis of disease resistance have focused on qualitative resistance and they attempted to find quantitative differences. A functional genomics approach was tested in which a CATMA whole-genome microarray was used to identify changes in gene expression associated with partial quantitative resistance in *A. thaliana* - *P. brassicae* interactions. The results showed an increasingly complex host response, as was the differential influence of *P. brassicae* infection on the transcription of *A. thaliana* genes according to the isolate used. They showed that, at the transcriptomic level, the partially resistant host decreased or delayed metabolic changes caused by the pathogen, induced earlier and/or stronger traditional plant defence responses, and actively inhibited cell enlargement and proliferation compared with the susceptible host.

Plant hormones are involved during the development of symptoms of clubroot (Ludwig-Muler and Schuller 2008). *A. thaliana* lines with low levels of auxin and cytokinin are more tolerant to clubroot, suggesting the development of galls may be limited or reduced by modifying levels of plant hormones (Grsic-Rausch et al. 2000; Siemens et al. 2006). Microarray data indicates that during infection by *P. brassicae*, most of the defence genes are either not expressed or are even down-regulated in the roots of *A. thaliana* (Siemens et al. 2006). These observations provide good theoretical bases on which to build functional defence systems in plant breeding by targeting differential reactions between resistant and susceptible plants.

1.8.2. Cultural controls

To optimise disease control, it is essential to create an environment less beneficial for disease development using cultural management methods. These include implementation of farm hygiene practices, rotating *Brassica* production with growth of non-host crops, elimination of cruciferous weeds and improving drainage. Additionally, it is important to monitor the level of infection in crops and know the concentration of spores in the soil prior to planting so that management decisions can be made that keep concentrations of *P. brassicae* in the soil below the threshold for disease (Friberg et al. 2005).

In Australia, to help farmers reduce the severity of the disease, guidelines have been developed for growers, nurserymen and contractors detailing strategies for the quarantine of new infections, disinfection and the production and distribution of all *Brassica* transplants (Porter and Cross 1995). Anecdotally, these guidelines have slowed the spread of clubroot within isolated growing regions. *P. brassicae* has been detected in many places where it is expected, e.g. irrigation pond sediment and dams (Datnoff and Lacy 1984; Faggian 2002) and in some where it is not, e.g. bores and *Brassica* seedling nurseries (Faggian et al. 1999).

The use of cultural methods is directed towards creating conditions unfavourable for disease development. This can include a wide range of crop production practices including farm hygiene, optimum plant spacing to reduce relative humidity around plants, provide good soil drainage through proper soil preparation and irrigation practices, the use of mulches to physically isolate above-ground plant parts from contact with the soil, and optimising fertiliser use to prevent stressed or overly succulent plants. As clubroot is a soil-borne disease, cultural practices that reduce the build-up of inoculum in the soil or create conditions unfavourable for infection or spread of inoculums are most useful for reducing the impact of clubroot.

Crop rotation is one of the oldest and most widely practised methods of managing soil-borne diseases. In Japan, many farmers still report significant crop losses due to clubroot in spite of their use of large quantities of chemicals to control the disease (Yamada, 2008). There the cultivation of sweetcorn as a break crop over a three-year period suppressed clubroot but did not provide complete control. Likewise, rotation of cabbage and sweetcorn resulted in a decline in the incidence of clubroot. Three or four-year rotations for cabbage cultivation in fields with severe outbreaks were recommended (Yamada, 2008). Sugino (2008) supported

the above recommendation, stating that crop rotation may be a useful method to mitigate clubroot in West Java, Indonesia. The use of bean, maize and potato in the fourth cropping season was recommended as better than continuous cabbage mono-cropping.

1.8.3. Chemical control

Agricultural chemicals, including fungicides and fumigants, can be used as part of an integrated strategy to manage soil-borne pathogens.

Much research effort has been directed towards controlling clubroot through the use of chemicals. In Australia and New Zealand, an integrated approach using disease prediction, farm hygiene, manipulation of soil pH and the use of lime and the fungicide fluazinan has been adopted (Cheah 1995; Falloon et al. 1997; Donald et al. 2006b). Much of this work was summarised in a review by Donald and Porter (2009) which details the benefits of integrated use of a range of treatments for the control of clubroot.

Pre-plant fumigant application in some cases is often a highly successful means of reducing soil-borne inoculum, although fumigant use is expensive and strictly regulated. The withdrawal of methyl bromide, the most widely used fumigant, for field use was necessary to reduce damage to the ozone layer in the Earth's atmosphere (Narisawa et al. 2000). Many other fumigants are also under review (Jaschke et al. 2010). Zuzak et al. (2013) used Vapam (metam sodium: sodium methyldithiocarbamate) as a soil fumigant for control of clubroot in canola in Canada and found that it effectively reduced clubroot severity at certain concentrations, but it is not registered for use with *P. brassicae* in all states and territories in Australia.

Seed sterilisation is always necessary to ensure that planting stock is disease-free, especially in seedling nurseries, as Donald (2005) stated that resting spores of *P. brassicae* remained viable in water for 34 months and repeated irrigation with water containing as few as 10 spores/mL resulted in root galling. Chlorine (in the form of hypochlorite) was effective. Datnoff et al. (1987) reported that 2 mg chlorine/L water reduced clubroot in cabbage and assessment of a collection of commercial disinfectant products available in Australia found that the most effective product against *P. brassicae* was hypochlorite (1000 ppm) (Donald et al. 2002).

Fungicide-treated seed is an important means of reducing the impact of seed and seedling diseases in many crops but its effectiveness against *P. brassicae* is limited because it is not seed-borne. Previous treatments such as dipping roots of seedlings in mercuric salt solutions at transplanting were effective but have been discontinued on environmental and health grounds.

Many studies have been conducted over a 70-year period to understand the interactions between calcium cyanamide and *P. brassicae* (Dixon and Wilson 1983; Coulshed and Dixon 1990; Dixon 2009b; Dixon and Williamson 1985; Donald et al. 2002; Donald et al. 2004b; Donald 2005). Donald et al. (2005) demonstrated that degradation of calcium cyanamide is associated with reduced viability of *P. brassicae.*

Pasold and Ludwig-Müller (2013) studied the reduction in clubroot formation in *A. thaliana* after treatment with prohexadione calcium (ProCa). They hypothesised that flavonoids could reduce clubroot development because prohexadione-calcium inhibits ascorbic acid/2-oxoglutaric acid-dependent dioxygenases such as flavanone-3-hydroxylase. The flavonols quercetin and kaempferol reduced the developments of galls, while the precursor naringenin accumulated, with the result that root systems treated with ProCa were better developed although galls were still noticeable. Also, they showed that the effect of ProCa treatment on clubroot reduction was not through changes in auxins. Lastly, chlormequatchloride (CCC) did not affect disease symptoms, which indicated that the reduction in infection was not because gibberellin biosynthesis was inhibited by ProCa.

Stimulation of the defence pathways via chemical or biotic signals that can induce plant defence responses and promote systemic acquired resistance (as recorded in other host-pathogen systems) may confer tolerance of or resistance to *P. brassicae* (Conrath et al. 2001). This has been investigated by several workers with mixed results. Salicylic acid and its functional analogue BTH did not reduce root galling in *B. rapa* (Ludwig-Muller et al. 1995). However, Agarwal (2009) demonstrated a reduction in root galling in *A. thaliana* following exogenous application of salicylic acid. David et al. (2013) also showed that exogenous salicylic acid at $\geqslant$5 mM significantly increased both shoot and root weights and reduced galls by 25-66% at 6 weeks after inoculation; also, a combination of salicylic acid and jasmonic acid reduced severity of clubroot by 25–35%. These approaches could lead the way to new chemical treatments based on naturally occurring compounds.

1.8.4. Biological control

There have been several attempts to find and develop biological methods (e.g. bacteria or fungi) for control of clubroot disease (Djatnika 1991; Einhorn et al. 1991; Elsherif and Grossmann 1991) and to use trap crops to reduce the soil inoculum load (Harling and Kennedy 1991) (for the latter, see next section) (**Table 1.10**).

Endophytic fungi colonise their host plant tissues without any visible symptoms of disease and do not alter the growth of host plants but they can protect their host plants against other pests and pathogens. It is thought that the presence of the endophytic fungus stimulates the plant defence system and salicylic acid is produced as a second messenger that activates many defences in the plant. A chytrid and several fungi have been suggested.

Acremonium species have been reported with antifungal activity against other pathogens and it was suggested that they might be useful as biological control agents for *P. brassicae* (Pieterse et al. 2002; Wilson 1995). Jaschke et al. (2010) found that inoculating with the endophytic fungus *Acremonium alternatum* reduced root galling in *A. thaliana*. Co-inoculation with *P. brassicae* reduced gall formation by about 20% and reduced the disease index (DI) by up to 50%. They proposed that inoculation with *A. alternatum* delayed the development of *P. brassicae* and confirmed this using quantitative real-time PCR (qPCR)

Table 1.10: Biological control methods with reported efficacy against *P. brassicae.*

Activity or organism	***Reference***
***Acremonium* spp.**	Jaschke et al. (2010)
Clonostachys rosea	Lahlali and Peng (2013)
Heteroconium chaetospira	Narisaw et al. (1998; 2000)
***Trichoderma* spp.**	Cheah et al. (2001)
Actinomycetes	Lee et al. (2008)
***Streptomyces* sp.**	Kim et al. (2002)
***Bacillus subtilis* XF-1**	Li et al. (2013)
Bait crops (oilseed rape)	Harling and Kennedy (1991)
Compost (green waste)	Tilston et al. (2002)
Furnace slag	Murakami et al. (2004)

to monitor the expression of differentially expressed *P. brassicae* genes. The authors showed that a compound released from spores was inhibitory to *P. brassicae* and suggested that this compound or the fungus could be the basis of a biological control programme.

Lahlali and Peng (2013) investigated the use of the biofungicide Prestop® (*Clonostachys rosea*) and the mechanism for its control of clubroot on canola. Applications of the biofungicide showed little effect on viability or germination of resting spores but did suppress clubroot on plants. Double applications of the biofungicide at 7-14 days after seeding showed greater clubroot control than a single application at either the seeding or post-seeding stage. It was hypothesised that, as the biocontrol fungus occupied the rhizosphere and interior of canola roots widely, it perhaps induced plant resistance based on up-regulation of the genes for jasmonic acid (JA-BnOPR2), ethylene (BnACO), and phenylpropanoid (BnOPCL, BnCCR) biosynthesis.

Narisawa et al. (1998, 2000) demonstrated that the endophytic fungus *Heteroconium chaetospira* reduced clubroot development in Chinese cabbage seedlings by 52–97%. It colonised the cortical cells of the host plant and significantly reduced the incidence of clubroot. It was effective as a biocontrol agent against clubroot in Chinese cabbage at low to moderate soil moisture and a pathogen density of $\leq 10^5$ resting spores per gram of field soil.

Trichoderma spp. protect plants and limit pathogen populations. They produce many biologically active compounds, including cell wall degrading enzymes and secondary metabolites, have been widely studied and are marketed commercially as biopesticides, biofertilisers and soil amendments (Cheah et al. 2000). Ghisalberti and Sivasithamparam (1991) and Vinale et al. (2008) suggested that they could be used to control clubroot. Cheah et al. (1997, 2000, and 2001) demonstrated that crushed maize colonised with *Trichoderma* spp. (TC32, TC 45, TC 63 and TC 64) or *Streptomyces* sp. (S99) reduced clubroot disease severity in cauliflower, although the mechanism was unknown. Although galling was reduced, there was no significant increase in top weight, unlike with drenching with benomyl.

Actinomycetes or their compounds have often been used in biological control. Kim et al. (2002) reported that *P. brassicae* could be controlled in *vivo* by some strains of *Streptomyces*. In particular, the study revealed three strains of *Streptomyces* effective in clubroot control: BG2- 17, BG2-19, and BG2-43. In a Korean study, Lee et al. (2008) reported 42-58% control of *P. brassicae* using endophytic actinomycetes. The effective strains were identified as *Microbispora rosea* subsp. *rosea* (A004 and A011) and *Streptomyces olivochromogenes*

(A018). They emphasised that for further verification efficacy trials must be conducted in the field.

Bacillus species have also been used in biological control. Li et al. (2013) examined the ability of fengycin-type cyclopeptides from *Bacillus subtilis* XF-1 to control clubroot disease. Twelve hours after treatment, the resting spores collapsed and by 24 h almost no cells were left intact. The conclusion was that the cyclopeptides cleaved the spore wall and could be developed as a mechanism for clubroot control.

Stringent regulations, particularly in Australia, have limited the number of registered biological control agents. The greatest limitation to biological control of plant disease has been variability in disease suppression. This is due to factors like host plants, soil chemistry, fertiliser application, biotic factors and rhizosphere ecology. Knowledge of how these and other factors affect BCAs can be used to increase the effectiveness and consistency of biocontrol. One advantage of using microbes as biocontrol agents is their ability to produce numerous secondary metabolites, many of which are potent antimicrobial allelochemicals.

Several factors could limit the use of microbial agents for biological control of plant pathogens. The most important of these are the survival in the soil between crops and in formulations (Walsh et al. 2001; Weller 2007; Weller et al. 2002), safety issues in human health and product formulation. In spite of these problems, biological control, if established, could provide a means of long-term control of clubroot.

1.8.5. Bait Crops

The practice of using a bait crop to induce resting spore germination in advance of a horticultural *Brassica* crop may offer a means of biological control of clubroot disease (Harling and Kennedy 1991). A glasshouse experiment conducted by Harling and Kennedy (1991) used seedlings of oilseed rape as a bait crop in soil inoculated with *P. brassicae*. These seedlings were collected at 2-10 weeks after planting in Chinese cabbage and subsequently clubroot declined by 60-95%. A subsequent field trial showed that the maximum reduction in galling was 25% when the bait crop was planted 4 weeks before the crop (Harling and Kennedy 1991). This contrasted with results from a field experiment by Ahmed et al. (2011), who showed only small resting spore populations differences between cruciferous, other host and non-host bait crops.

1.8.6. Solarisation

Soil solarisation is a technique of heating soil by covering it with transparent polythene sheeting during hot periods to control soil-borne diseases. Soil solarisation has been used in Japan since 1980 to reduce the inoculum loads in heavily infested fields (Horiuchi 1991; Yoshikura et al. 1986). This method has been used commercially for growing high-value crops in diseased soils in environments with a hot summer (Katan 1992). In Australia the method would only be economic to use on infested farms during periods of high temperature, in particular in January and February. Solarisation is considered safe because of the lack of chemical materials and residual compounds. However, Horiuchi (1991) argued that soil solarisation under field conditions did not provide sufficient control of clubroot.

In Australia, Porter et al. (1991) showed that solarisation, combined with low doses of the soil fumigants dazomet at 100 kg/ha and methyl bromide at 100 or 250 kg/ha, provided significant control of clubroot and increased yield of cauliflower in field trials at Werribee and Keysborough. This method has been used to control clubroot effectively during the summer months in Australia (Porter et al. 1991), although the method is not widely practised commercially due to its cost and the practical issues of laying, lifting and disposing of plastic (Donald 2000), though it could be important in organic farms.

1.9 Scope of this study

Understanding variation in the pathogen and its effects on pathogenicity is fundamental to the ability to manage clubroot disease. Traditionally this variation has been studied using a range of differential sets of *Brassica* host plants. Such studies are time-consuming and their outcomes influenced by environmental factors and limited by the genetics of the differential hosts available. In addition, all such tests are unable to differentiate mixed pathotypes within a single root gall. This study sought to study variation in the pathogen by molecular methods to investigate if they could offer a more rapid and reliable means of differentiating virulence in the pathogen population. The study sought to answer the following questions:

1. **Can a molecular assay be developed to differentiate pathotypes among strains to replace plant-based assays?**
2. **What differences are observed in pathogenicity with variation in virulence in the pathogen and resistance in the host?**

Chapter 2. Assessment of pathotype diversity in Australian populations

2.1 Introduction

Clubroot is mainly spread by movement of the resting spores of *P. brassicae* via contaminated soil or water or by infected seedling transplants. Dams, drainage or flood waters can become contaminated with run-off from clubroot-affected land and spread *P. brassicae* from field to field or over much wider distances. Likewise, soil contaminated with resting spores of *P. brassicae* is readily spread on farm equipment and machinery (in particular tillage tools), on the hooves of grazing animals, as dust carried by wind and, in some cases, on seed of crucifer crops as well as hay and straw, which can become contaminated with resting spores via dust when they are grown next to clubroot-infested fields (Zitter 1985; Walker 1952; Porth et al. 2003).

The degree of damage caused by *P. brassicae* to the crop depends on the degree of infestation and the virulence of the pathogen as well as the resistance of the host plant. Some pathotypes are less virulent than others and cause little damage even if infestation is high, whereas others cause much more severe damage and even up to 100% crop losses (Some et al. 1996; Kuginuki et al. 1999; Manzanares et al. 2001; Strelkov et al. 2006; Strelkov et al. 2007; Cruz et al. 2008; Cao et al. 2009).

2.1.1 Assessment of pathogenicity and virulence

Variation in pathogenicity and virulence of pathogen populations shows that the pathogen is adaptable to changing ecological conditions, giving it more chance of infecting a wide range of hosts. In the following, the term ‘pathogenicity’ is used to denote the ability of a pathogen to infect a host, while the term ‘virulence’ is used to denote the ability of the pathogen to cause a varying degree of damage to the host.

Studying pathogenicity and virulence as classic concepts can provide a successful model for analysing the role of microorganisms in disease production. Also, *P. brassicae* has pathotypes (i.e. the pathogen displays host specificity) in Australia or worldwide (Toxopeus et al.1986; Kuginuki et al. 1999; Donald et al. 2006a; Dixon 2009a); this has been problematic in the search for resistance genes due to conflicting reactions on the host species, as was predicted from earlier research (Appel and Werth 1910).

Honig (1931) was one of the first researchers who gave a practical demonstration showing the existence of the concept (Lammerink 1964; Karling 1968). He wrote that there had been much study of the physiological characterisation of *P. brassicae* many decades previously but there was uncertainty and difficulty in comparing the information because of the deficiency of cross-referencing and lack of rationalisation between methods. Since then, numerous sets of differential hosts have been proposed for the assessment of virulence in the pathogen (Ayers 1957; Williams 1966; Buczacki et al. 1975; Some et al. 1996). Each system has its advantages and disadvantages.

Several sets of differential hosts are needed to classify *P. brassicae* races according to their plant reactions, in terms of gall formation and size (Williams 1966; Karling 1968). The pathotypes are identified by their virulence on a host differential set, which is a group of host plants that serves to distinguish between various pathotypes of a pathogen based on disease symptoms they cause on a defined group of hosts.

Over time, numerous differential sets have been proposed to identify clubroot pathotypes. There are two classic methods commonly used to differentiate populations of *P. brassicae*, the first being differential hosts, developed by Williams (1966), and the other being a subsequent development, the European Clubroot Differential (ECD) series proposed by Buczacki et al. (1975). This system represented considerable advances over other systems and can be considered as the result of several previous attempts and independent research groups, as well as being relatively cheap and simple to set up. All systems do, however, need laboratory experience and are time-consuming and subjective as they depend on the host plant's outer appearance, requiring some degree of potentially subjective observation.

Three differential sets are now most commonly used:

1-Williams differential set (1966)

The Williams differential set for the determination of races of *P. brassicae* that infect two host species consists of two cabbage (*B. oleracea*) cultivars (Danish Ballhead and Badger Shipper) and two rutabagas (*B. napobrassica*) (turnip or yellow turnip) cultivars (Laurentian and Ditmars S2)(Ayers 1972). The advantages of this set are that the observations are straightforward and use only a small set of hosts. The disadvantages are that it uses a limited number of host species and cultivars. It was developed to identify pathotypes of pathogens isolated from cabbage and rutabaga only; moreover it depends on observations that are subjective and liable to misinterpretation. It is, however, still widely used.

2-European Clubroot Differential Set (1975)

In 1975, Buczacki et al. developed a broader international standardised test symptom grading scale, thus making it both broadly accessible and useful. It has been internationally accepted and is routinely used by many researchers (Donald et al. 2006a; Strelkov et al. 2006; Kasinathan 2012;). This set comprises 15 differential *Brassica* hosts in three subsets:

- *B. rapa* (5 hosts)
- *B. napus* (5 hosts)
- *B. oleracea* (5 hosts)

Buczacki et al. (1975) determined that within these series there were different numbers and kinds of resistant genes. The bioassay built on the relative susceptibility of the differential hosts and virulence of the clubroot populations, by comparing the effect of the pathogen on a more extensive set of standard host plants. This system has been extensively used by many researchers in this area: Dobson et al. (1983), Toxopeus et al. (1986) and Donald et al. (2006a), to differentiate *P. brassicae* populations across the west coast of the USA, Western Europe and Australia respectively.

The advantages of this set are that it has a wide range of hosts and that the information can be observed on multiple species; therefore it enables broader comparisons than Williams' set. It does, however, have some disadvantages, in that it is easily influenced by environmental conditions and badly affected by the loss of host plants, not all hosts are differential and it has a complicated strain nomenclature (this is discussed more later on). It has been widely used in Australia and adopted by the Victorian Department of Primary Industries (Donald et al. 2006a), among others (Kong Kaw Wa 2009).

3-Differential set of Some et al. (1996)

Some et al. (1996) developed another set to identify pathotypes to study the variation in virulence on *B. napus* among *P. brassicae* collections from France and derived single-spore isolates. This set consists of three *B. napus* hosts and has some advantages. Similar to the Williams (1966) sets, it is more straightforward and consists of a small set of hosts based on reactions of cultivars of *B. napus*. Its disadvantages are that it has a limited number of species on which it is differentiated, there is a chance to miss pathotypes, it has low differentiating capacity and results cannot be generalised to all *Brassica* groups (Some et al. 1996).

Recently, Strelkov et al. (2012) have developed a new clubroot differential set, the 'Canadian Clubroot Differential' (CCD) set, by using a phased procedure to develop a differential set of

hosts for *P. brassicae* from canola. In brief, this focuses on *B. napus* genotypes with good differentiating capacity, but also includes some key *B. rapa* genotypes and excludes *B. oleracea*. This has not been widely used so far in Australia but has potential for pathotypes from canola.

As the most commonly used method of determining pathogenicity and virulence is the ECD, this was adopted in this thesis and is further detailed here.

2.1.2 How does the European Clubroot Differential (ECD) series work?

Root galls are collected from infected root tissue. A resting spore suspension of *P. brassicae* is prepared from each collection of root galls and is used to inoculate the differential hosts of the ECD series. These hosts are then grown in sterilised soil under controlled conditions for a set period of time, fertilised and watered as appropriate to maximise growth potential, and then the root systems are excavated and the degree of galling scored on a numerical scale that assigns greater numbers to galling on the main tap root rather than the lateral roots. As soon as the differential interaction is documented, the *P. brassicae* sample is classified as a differentially interacting population or simply, a 'population' (Buczacki et al. 1975).

These *P. brassicae* samples frequently involve a mixture of separate populations, each capable of a differential interaction. Therefore, it is likely that populations derived from first inoculations can be subdivided into additional populations when resting spores are extracted from developing galls and re-differentiated onto the ECD series (Some et al. 1996; Manzanares et al. 2000a). In addition, there are many factors involved in discriminating *P. brassicae* populations, and so to investigate and deal with pathogens like *P. brassicae*, there are rules and instructions for the collection, storage, extraction and application of the clubroot isolates when conducting the ECD tests (Buczacki et al. 1975).

As with any agricultural experiments, as well as the physical conditions of the soil and the spore concentration, several environmental factors such as temperature, soil moisture content, nutritional elements and ion concentration are likely to have an effect on the level of clubroot infection and development (Karling 1968).

To provide the most favourable conditions for the successful invasion and proliferation of *P. brassicae* resting spores during the test, in all experiments the potting mix is treated according to Yoshikawa (1981) and high moisture levels are maintained throughout the

experiment. In accordance with Manzanares et al. (2000a), other factors are controlled through the regular application of liquid fertiliser and by growing the *Brassica* hosts in environmentally controlled glasshouses/growth rooms, while the preparation and application of clubroot isolates is carried out to optimise the reproducibility, reliability and cross-referencing of the data for the analysis.

2.1.3 Terms used

Since there are many terms that refer to the identification, composition and particular forms of the pathogen, many researchers have suggested numerous expressions to avoid misinterpretation, although some of these have been employed haphazardly in the clubroot literature. The most important of these terms are below.

A collection is a term commonly used during the study of characterisation of *P. brassicae* and it refers to resting spores mainly recovered from infected root galls (clubs) that are used as inoculum to inoculate healthy or intact plants during the experimentation process. Field collections are frequently made up of a number of separate populations, each capable of differential interaction (Buczacki et al. 1975). By contrast, the term 'single spore isolate' is defined as a population derived from a gall inoculated with a single resting spore and maintained in isolation (Vorrips 1995).

To agree with the suggestions of Voorrips (1995) and Crute et al. (1980) in their studies, the term 'pathotype' will be used throughout this study instead of the term 'race' regardless of the author's original terminology. This accords with several other authors who have also suggested that it is preferable to use the term 'pathotype' than to use 'race' (Buczacki et al.1975; Crute 1980; Voorrips 1995; Xue 2008).

In addition, Buczacki et al (1975) emphasised that the term 'physiological race' may only be used when further subsequent extractions and differentiations of a population provides similar results, thus indicating a homogenous population. Toxopeus et al. (1986) stated that in Canada, Germany, Netherlands, England and the United States, at least nine such physiological races or pathotypes of *P. brassicae* are known. In Australia, Donald et al (2006a) found that 41 collections of *P. brassicae* from important vegetable *Brassica* production regions in Tasmania, New South Wales, Queensland, Western Australia and Victoria generated 23 triplet codes. The reproducibility of this data is unknown and it may be assumed that at least some of the collections used in this study were made up of mixed

populations. The pathotype 16/31/31 was not found in these studies; the most common pathotypes in Australia were those with triplet codes 16/3/12 and 16/3/31 (Donald et al. 2006a). These were closer to populations of *P. brassicae* reported from the USA than those from Europe.

2.1.4 Aims of this chapter

The aim of this thesis was to examine virulence in some Australian populations of *P. brassicae* using a combination of cultural, molecular and morphological techniques. The starting point was to examine the phenotypes of the pathogen, by estimating the variation in virulence between different pathotypes of the pathogen across Australia. Identifying pathotypes of *P. brassicae* in Australia and worldwide could contribute ultimately to the development of clubroot resistance in brassica crops (Toxopeus et al. 1986; Kuginuki et al.1999; Donald et al. 2006a).

The aims of this chapter were the following:

1. To collect, identify and characterise pathogenicity and virulence in *P. brassicae* populations from across Australia.

2. To use these characterised populations to determine if there was any relationship with their sources (geographically).

3. To determine if there were pathotypes that had not been reported in previous studies in Australia or throughout the world.

4. To use these characterised populations subsequently to characterise them molecularly to see if molecular determinants of virulence could be identified (Chapter 3).
5. To use these characterised populations subsequently to characterise morphological differences in gall formation between hosts and pathogens with different degrees of resistance and virulence (Chapter 6).

2.2 Materials and Methods

2.2.1 Pathogen collection

2.2.1.1 Collecting fresh samples from a farm

A field trip was undertaken to Woori Yallock, Victoria, Australia, to a farm identified as suffering from clubroot disease outbreaks (37.8°S, 145.5°E). The farm had clubroot on *Brassica* crops at a higher elevation but not at a lower elevation. Several infected volunteer broccoli plant roots were collected at the higher elevation and spores isolated from these were used to inoculate the ECD hosts; they were also used to test for *P. brassicae* DNA using universal and specific primers and investigated for genetic variation (Chapter 3).

2.2.1.2 Other sources of root galls

The remainder of the collections of *P. brassicae* were donated as naturally infected galled plants from *Brassica* vegetables from farms located in Victoria (VIC) and Western Australia (WA) as fresh and frozen root gall samples provided by Dr Caroline Donald (Department of Primary Industries, Victoria (DPI VIC) (**Table 2.1, Fig 2.1**). Samples were cleaned of soil and washed with tap water and dried. Galls (infected roots) were preserved frozen in clean plastic bags and then placed in in a standard freezer (-18 ºC ~ -25 ºC).

Table 2.1: Populations of *P. brassica*e collected, their locations and collection dates.

Population No.	**Location (letters in brackets refer to Fig 2.3)**	**Latitude (°S)**	**Longitude (°E)**	**Date of collection**
1	Boisdale – Victoria (a)	-37.88032	146.98730	10/04/2002
2	Woori Yallock – Victoria (f)	-37.77917	145.53074	24/05/2009
3	Devon Meadows – Victoria (d)	-38.16199	145.30202	4/04/2008
4	Mornington (Rosebud) - Victoria (c)	-38.36102	144.89005	28/01/2008
5	Trentham- Victoria (h)	-37.38892	144.32286	23/04/1999
6	Cora Lynn - Victoria (e)	-38.14413	145.61139	13/09/1999
7	Werribee – Victoria (g)	-37.90291	144.65847	16/04/1998
8	Lindenow- Victoria (b)	-37.79540	147.46988	18/03/2008
9	Manjimup - Western Australia (i)*****	-34.24184	116.14559	4/11/1998
10	Manjimup – Western Australia (i)*****	-34.24184	116.14559	7/01/1999

***** Two different properties and property owners (these samples were not from the same property).

Figure 2.1: Sites where naturally infected *Brassica* crops were collected: a. Boisdale, VIC; b. Lindenow, VIC; c. Rosebud, VIC; d. Devon Meadows, VIC; e. Bunyip, VIC; f. Woori Yallock, VIC; g. Werribee, VIC; h. Trentham, VIC; i. Manjimup, WA. **(http://maps.google.com.au & http://universimmedia.pagesperso-orange.fr/geo/loc.htm**

2.2.2 ECD testing

2.2.2.1 Preparation of spore suspensions from infected plants

A total of 10 clubroot samples was investigated, each originating from a frozen gall. Spore suspensions were prepared by thawing the galls and macerating them using a mechanical blender with three times their volume of sterile distilled water (sdH_2O) (1:3 w/v) at high speed. The homogenate was filtered through four layers of sterilised muslin. The resulting spore suspensions were stored at 4°C (Castlebury et al. 1994).

For viability studies, it was recommended to clean spore suspensions further, as galls might contain additional plant cell debris and contaminating bacteria, and so 30 mL of resting spore suspension was measured into a 50 mL centrifuge tube and centrifuged at 3,120 x g in a bench-top centrifuge (Centaur 2, Stansens MSE) for 10 min. The supernatant was discarded and the pellet resuspended in to 45 mL sdH_2O and stored at 4ºC. Aliquots were examined microscopically to show that bacterial contamination was minimal. For each sample all of these steps were repeated at least three times for each suspension.

2.2.2.2 Quantification of resting spores in suspension

Resting spores were counted using a Neubauer haemocytometer and the concentration of the isolates was adjusted to 10^8 spores mL^{-1} in MilliQ water before inoculation. These spore suspensions were stored at 4ºC for only a few weeks before use (Donald et al. 2006a)

2.2.2.3 Inoculation and growth of the ECD host plants and maintenance of screening studies

The 15 differential hosts of the ECD series comprising 15 *Brassica* lines were used. These comprised five varieties of each of three *Brassica* species: *B. rapa, B. napus and B. oleracea* (Buczacki et al. 1975), supplied by Horticultural Research International, Wellesbourne, England. The seeds were stored dry at 4°C until used (**Table 2.2**).

All seeds were surface-sterilised by soaking in 70% ethanol for 5 min followed by three rinses in sterile distilled water, and then immersion in 2% sodium hypochlorite solution for 10 min followed by three rinses in sterile distilled water for 5 min each time. To promote seed germination, seeds were placed in sterile plastic Petri dishes and placed at 18ºC for 24 h before germination (Smith and Price 1997; Taski-Ajdukovia and Vasic 2005).

Seedlings were transferred from Petri dishes and sown 2-3 mm deep in 10 cm diameter surface-sterilized pots (as for seeds) filled with autoclaved (three steam cycles at 105ºC for 60 min) General Purpose® potting mix (Yates™, NSW) that had its pH adjusted to ~6.5 with hydrated lime (Richgrow™). Pots were arranged in a randomised block design and consisted of four replicate pots, each containing three seedlings of each host (**Fig 2.2**). Each pot sat in a container to catch any leachate, which was later poured back into the pot.

All plants were watered every 2 days and grown at 20±5ºC in a glasshouse with natural light under a 16 h photoperiod (fluorescent lamps about 2 m above the plants) and ~70% humidity. The potting mix was kept moist throughout the experiment with liquid fertiliser (20 mL Nitrosol™/10 L tap water). No other chemical or pesticide was applied (Kong Kaw Wa 2009).

Pots were thoroughly watered immediately prior to inoculation at approximately14 days after germination. Each 'treatment' seedling was inoculated at the two-leaf stage by pipetting 1 mL of the spore suspension into a small depression in the potting mix at the base of the seedling (**Fig. 2.3**). For comparison, one pot of four plants for each ECD line was used as a control per trial to check for the non-appearance of infection; for these, 1 mL of sterile MilliQ water was applied to the 'control' seedlings and the control pots were placed in a black plastic crate. Inoculated plants were checked for infection every 3 days (Donald et al. 2006a).

2.2.2.4 Disease assessment of the ECD hosts

For disease assessment, after 6–8 weeks, plants were removed from their pots, the roots washed free of potting mix and the degree of clubroot symptoms visually rated for clubroot severity on a 4-point scale as follows: 0 no visible root galls; 1 less than 10% roots visibly galled; 2 between 10 and 50% of roots visibly galled and 3 greater than 50% of roots visibly galled (**Figure 2.4**).

Dobson et al. (1983) suggested a formula for assessment and disease index (DI). Disease index was calculated for each host and used to assign one of three host reaction types (resistant DI = 0, indeterminate 0 < DI < 33 and susceptible DI ≥ 33) as shown in the formula below:

$$\mathbf{DI = (1}n_1 \mathbf{+ 2}n_2 \mathbf{+ 3}n_3\mathbf{).100/3}N_t$$

where n_1 to n_3 is the number of plants in the indicated class and N_t is the total number of plants tested. Differential hosts with DI = 0 were considered resistant and susceptible ones had a DI ≥ 33, while those between these values had indeterminate resistance.

Table.2.2: The European Clubroot Differential (ECD) series with associated host numbers and denary values (after Buczacki et al. 1975, with scientific names after Crute et al. 1980).

Differential number	**Differential Host**		**Denary value**
	Variety and cultivar or line	**Common name**	
	20 chromosome group (*Brassica rapa*)		
01	var. *rapifera* line aaBBCC	Fodder turnip	1
02	var. *rapifera* line AAbbCC	Fodder turnip	2
03	var. *rapifera* line AABBcc	Fodder turnip	4
04	var. *rapifera* line AABBCC	Fodder turnip	8
05	var. *chinensis* cv. Granaat	Chinese cabbage Pe-Tsai	16
	38 chromosome group (*Brassica napus*)		
06	var. *napus* line Dc101	Fodder rape Nevin	1
07	var. *napus* line Dc119	Giant rape commercial	2
08	var. *napus* line Dc128	Giant rape selection	4
09	var. *napus* line Dc129	New Zealand resistant rape	8
10	var. *napus* line Dc130	Swede Wilhelmsburger	16
	18 chromosome group (*Brassica oleracea*)		
11	var. *capitata* cv. Badger Shipper	Cabbage	1
12	var. *capitata* cv. Bindsachsener	Cabbage	2
13	var. *capitata* cv. Jersey Queen	Cabbage	4
14	var. *capitata* cv. Septa	Cabbage	8
15	var. *fimbriata* cv. Verheul	Fimbriate kale	16

Figure 2.2: European Clubroot Differential (ECD) series placed in greenhouse**.**

Figure 2.3: 'Pipette' method used to inoculate seedlings with *P. brassicae* spore suspension.

2.2.2.5 Denary values for each population

The clubroot populations were characterised using the denary values of the susceptible differential hosts (Buczacki et al.1975; Crute et al. 1983; Toxopeus et al.1986; Donald et al. 2006a). For instance, if ECD hosts 05, 12, 13 and 14 were susceptible (**Table 2.2**), the ECD

code for this isolate would be 16/00/14, i.e. the addition of the denary values of the susceptible hosts for each *Brassica* group. Only those pathotypes of *P. brassicae* causing a DI ≥80 on at least one of the ECD hosts were analysed in this study. This arbitrary value, used by Crute et al. (1983), Toxopeus et al. (1986) and Donald et al. (2006a) was adopted to ensure that the data reported were obtained from infective and highly viable clubroot inoculum**.**

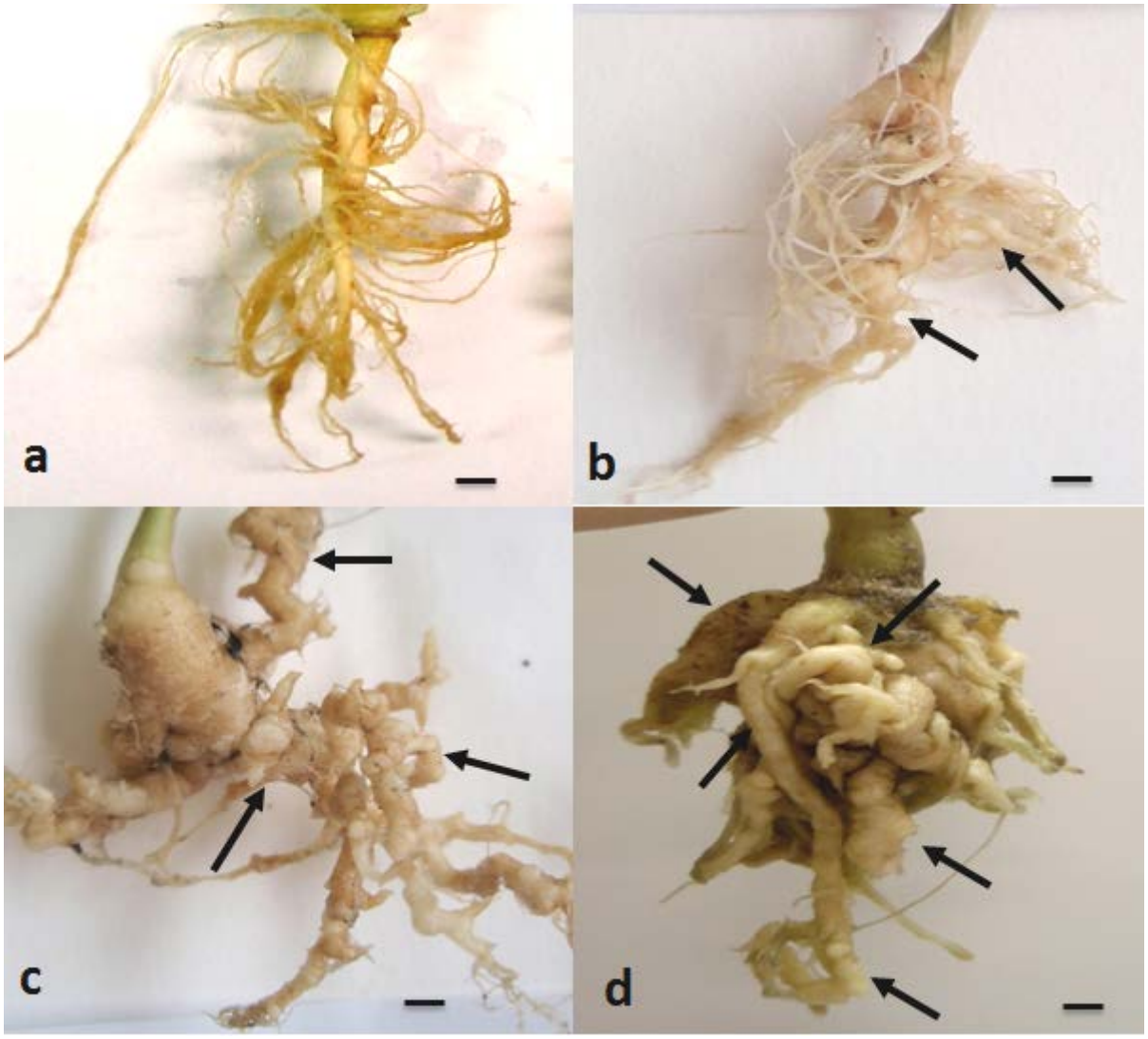

Figure 2.4: The 4-grade scale used to assess clubroot symptoms. (a) 0 = no visible clubbing, (b) 1 = less than 10% roots visibly galled, (c) 2 = between 10 and 50% of roots visibly galled, (d) 3 = 3 greater than 50% of roots visibly galled. Horizontal black scale bars represent 8 mm.

2.3 Results

All ten *P. brassicae* populations produced galls on at least one of each of the 15 ECD hosts and produced a different response for each ECD host plant. Ten triplet EDC codes were thus identified from ten populations, each population representing a different ECD code (**Tables 2.3; 2.4**). The majority of the populations infected all three species comprising the ECD hosts. Except for population 10, each of the populations infected at least one of the five hosts in each of the three species, leading to triplet codes without zeros.

Scores for the *B. oleracea* hosts were much more variable (12-31) than those for the *B. rapa* hosts (uniformly 16) and the *B. napus* hosts (2-3). All 10 populations appeared virulent on the highly susceptible differential host *B. rapa* ECD05 (DI= 78.8-100%, average 88%) and two *B. oleracea* hosts, ECD13 (DI=75-100%, average 89%) and ECD14 (DI=75-100%, average 87%) as shown in Table 2.4.

Three populations (4, 7 and 8) were defined as highly virulent towards *B. oleracea* (this virulence is only towards *B. oleracea*, not all *Brassica* species) according to their ECD codes of 16/02/30, 16/03/30 and 16/03/31 respectively. Populations 1, 2, 3, 5 and 9 were intermediate in virulence. Population 10 was marginally more virulent than population 6 but did not infect any of the *B. napus* hosts. Population 6 was less virulent than the other populations because ECD11 and ECD12 were resistant, whereas they were susceptible to other populations. Populations 9 and 10 had been collected from the same locality but at different times and only differed in their pathogenicity to the *B. napus* hosts.

In the *B. rapa* hosts, there was no variation in the reaction of Australian pathogen populations. Only ECD05 (cv. Granaat) produced clear symptoms with all isolates tested. Virulence to *B. rapa* hosts was therefore limited.

The *B. napus* hosts had relatively little infection and hosts showed diverse reactions; the response varied from resistant to indeterminate and susceptible. ECD08, ECD09 and ECD10 were resistant but ECD10 plants had low levels of infection (DI was 0% and 19% against populations 5 and 4 respectively). ECD07 plants were susceptible to all populations except for population 10, which failed to infect and had DI=0%. ECD06 was intermediate between ECD07 and ECD08-ECD10. Resistance in this group was high.

In the *B. oleracea* hosts, all populations except population 6 succeeded in infecting and produced moderate-severe symptoms. Only ECD11 and ECD12 showed resistance and were only resistant to population 6. ECD13 and ECD14 were susceptible to all populations. Other hosts showed varying intensity of symptoms. Overall, resistance to most of these populations was restricted.

There were some differences between this and previous ECD codes for some populations as recorded formerly in 2006 (Donald et al. 2006a). There was no variation in the code (16) for *B. rapa*, minor variation (1-2) in the code for *B. napus* but large variation (15-31) in the code for *B. oleracea*. There was no trend with time.

Table 2.3: The reactions of the ECD hosts to Australian collections of *Plasmodiophora brassicae.*

Clubroot population ID	State of Australia	Property of origin[a]	Date of collection	Host[b]	Test host															Previous ECD codec	ECD code[d] (this study)
					Brassica rapa					*Brassica napus*					*Brassica oleracea*						
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1	VIC	Boisdale	2002	Cabbage	R	R	R	R	S	?	S	R	R	R	S	S	S	S	?	16/01/31	16/02/15
2	VIC	Woori Yallock	2009	Broccoli	R	R	R	R	S	?	S	R	R	R	?	S	S	S	?	NA	16/02/14
3	VIC	Devon Meadows	2008	Broccoli	R	R	R	R	S	S	S	R	R	R	S	S	S	S	?	16/0/15	16/03/15
4	VIC	Mornington	2008	Broccoli	R	R	R	R	S	?	S	R	R	?	?	S	S	S	S	16/03/15	16/02/30
5	VIC	Trentham	1999	Cabbage	R	R	R	R	S	S	S	?	R	R	S	?	S	S	?	16/03/12	16/03/13
6	VIC	Cora Lynn	1999	Cauliflower	R	R	R	R	S	?	S	R	R	R	R	R	S	S	?	16/02/30	16/02/12
7	VIC	Werribee	1998	Broccoli	R	R	R	R	S	S	S	R	R	R	?	S	S	S	S	16/03/12	16/03/30
8	VIC	Lindenow	2008	Cabbage	R	R	R	R	S	?	S	R	R	R	S	S	S	S	S	16/02/31	16/02/31
9	WA	Manjimup	1998	Broccoli	R	R	R	R	S	S	S	R	R	R	?	S	S	S	?	NA	16/03/14
10	WA	Manjimup	1999	Cauliflower	R	R	R	R	S	R	R	R	R	R	?	S	S	S	?	NA	16/00/14

[a]With the exception of the Mornington sample (population ID 4) original host material, i.e. that which was used for previous ECD testing, was used in this study. The Mornington sample was originally collected in 1996; material used in this study was from a second collection conducted during 2008 at the same site on the original property. [b]Original host material used to prepare the inoculum. [c]Previous test outcome obtained by Dr Caroline Donald (DPI Victoria, pre 2006 results reported in Donald et al. (2006a). [d]ECD codes obtained in this study and assigned on the basis of susceptible reactions only, where disease index was DI ≥80 on at least one susceptible host to be considered a valid ECD test. R, resistant (DI = 0); S, susceptible (DI ≥33); ?, indeterminate (0 < DI < 33) ; NA, ECD code unknown: the Woori Yallock sample had not been tested previously, both Manjimup samples were previously included in the work conducted by Dr Caroline Donald (DPI VIC) but in both instances a disease index >80 was not obtained for any host and therefore the data were not reported.

Table 2.4: Detailed assessment of clubroot symptom formations in the ECD series

ECD	***Plasmodiophora brassicae* population ID%**									
	Population 1	Population 2	Population 3	Population 4	Population 5	Population 6	Population 7	Population 8	Population 9	Population 10
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	83.33	91.7	94.4	100	80.55	80.55	88.88	86.11	100	78.88
6	22.22	27.8	36.1	16.66	66.66	13.88	100	13.88	66.66	0
7	58.33	77.8	75	69.44	72.22	77.88	100	69.44	88.88	0
8	0	0	0	0	15.15	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	19.44	0	0	0	0	0	0
11	83.33	25	86.11	11.11	84.84	0	25	100	25	5.44
12	80.55	45.5	77.77	86.11	25	0	88.88	100	45.5	45.5
13	88.88	86.1	86.11	100	90	88.88	100	86.11	88.88	75
14	75	86.1	84.84	100	86.11	77.77	91.66	100	77.77	86.11
15	30.55	13.33	30.88	44.44	25	5.55	80	77.77	30.8	25
ECD Code	**16/02/15**	**16/02/14**	**16/03/15**	**16/02/30**	**16/03/13**	**16/02/12**	**16/03/30**	**16/02/31**	**16/03/14**	**16/00/14**

Disease index (DI) calculated by **DI = (1*n1* + 2*n2* + 3*n3*).100/3*Nt*** where *n1* to *n3* is the number of plants in the indicated class and *Nt* is the total number of plants tested.

2.4 Discussion

2.4.1 Variation in pathotypes from Australia and the world

This study found one new pathotype of *P. brassicae* from ten collections in only two states of Australia: four new pathotypes from Donald et al. (2006a) and one from Kong Kaw Wa (2009). Such studies to define the range of contemporary pathotypes are essential to efforts in breeding for clubroot resistant plants because they help to define the virulence of the pathotypes to which resistance must be found. Moreover, as *P. brassicae* challenges *Brassica* crops by presenting different pathotypes and races over time, it is a constant challenge to devise multiple *Brassica* varieties with resistances to the wide range of contemporary clubroot populations.

Australian populations in general vary from those earlier reported from overseas (mainly Western Europe) in their deficiency of virulence to the *B. napus* group of hosts (Toxopeus et al. 1986). All Australian triplet codes are uniform in their virulence on *B. rapa* hosts (code of 16), low in virulence on *B. napus* hosts (code of 0-3) and high in virulence on *B. oleracea* hosts (code of 12-31). In general, there was little virulence in these pathogen populations for the *B. rapa* host group, except for the universally susceptible ECD05, while there was greater virulence for the *B. napus* host group and high virulence for the *B. oleracea* host group.

The large diversity of ECD codes recorded in this study from only two states in Australia (ten pathotypes from ten collections) is similar to the large diversity recorded in previous studies of Australian populations (Donald et al. 2006a; Kong Kaw Wa 2009). The true diversity in Australia may be greater than noted here, as no samples from other states were examined, unlike the case in the national study by Donald et al. (2006a). Frozen samples of galled roots were obtained from other states but failed to produce galling and were therefore excluded from this report.

Most denary codes found here have been recorded in previous Australian studies (Donald et al. 2006a), but one was new to Australia. The pathotypes 16/00/14, 16/02/14, 16/02/15, 16/02/30, 16/02/31, 16/03/14, 16/03/15 and 16/3/30 have been recorded in these former studies, while ECD code 16/02/12 was recorded for the first time in Australia, though this needs to be confirmed by further studies. This information may be useful for selection and/or breeding programmes at the national level or state level (Dobson 1983; Donald et al. 2006a; Kong Kaw Wa 2009).

The high diversity of Australian populations contrasts markedly with those from other countries. In 2006, an inclusive survey covering the most important vegetable *Brassica* production regions in Western Australia, Queensland Tasmania, New South Wales and Victoria generated 23 triplet codes from 41 collections of *P. brassicae* (Donald et al. 2006a). Kong Kaw Wa (2009) recorded six triplet codes from only seven populations of *P. brassicae* from Victoria and WA, and this study has generated ten triplet codes from ten populations. This contrasts with the much fewer triplet codes from Europe and North America.

Strelkov et al. (2006), in Canada, characterised seven *P.brassicae* populations from different provinces in Canada by the European Clubroot Differential (ECD) series. They identified four ECD codes for the seven populations: Alberta populations were classified as ECD 16/15/12 and 16/15/0 while the population from British Columbia was rated as ECD 16/2/12 and the Ontario population was classified as ECD 16/0/14. They indicated that the populations from Alberta were more virulent than the populations from the other regions (British Columbia and Ontario).

Bernard et al. (2006) investigated a collection of *P. brassicae* from two regions in the Philippines using the ECD series. Three different ECD codes were designated; the populations from La Trinidad provinces were classified as ECD 31/31/31 and ECD 19/31/31, while the Buguias population was designated as ECD 21/23/31.

In an earlier study in the USA, Dobson et al. (1983) found only two ECD codes (16/02/31 and 16/03/31) from 13 collections of *P. brassicae*, much fewer than expected. Similarly, Toxopeus et al (1986) found only nine pathotypes in Europe and North America. The pathotype 16/31/31 was not found in the Australian studies; the most common pathotypes in Australia being the triplet codes 16/3/12 and 16/3/31 (Donald et al. 2006a). These were closer to populations of *P. brassicae* reported from the USA than to those from Europe, where susceptibility in *B. napus* is common (discussed further in Section 2.4.1.2).

Strelkov et al. (2007) conducted a field survey for clubroot in the regions surrounding Edmonton, Alberta, Canada, in 2005. The results confirmed the presence of clubroot in 41 of the 112 canola (*B. napus*) fields surveyed. Also, ten populations of the pathogen were selected and used in pathotype classification by the European Clubroot Differential (ECD) set; all *P. brassicae* populations were classified as ECD -/15/12, which can be considered as low virulence. Moreover they showed no significant difference in the codes of the ten populations tested.

Most collections of *P. brassicae* tested here showed a large degree of similarity, as did those from Kong Kaw Wa (2009). Most of these collections were from Victoria and from reasonably close areas (**Table 2.5**). The distances between adjacent farms was 50.6-186 km (**Table 2.5**), suggesting local disease spread; after the disease appeared in one field, it probably spread by well-known factors such as movement of agricultural machinery and flooding to adjacent fields and properties. However, the two WA populations were collected from Western Australia and are a little different (16/00/14 as opposed to 16/03/14) in denary code for *B. napus*, perhaps as a result of environmental factors in the fields (Jones et al. 1982a; Donald et al. 2006a).

Table 2.5: Locations of and estimated distance between collection points in Victoria (Vic) and Western Australia (WA).

Location	The estimated distance
Boisdale ,Vic to Lindenow, Vic	57.6 km
Boisdale ,Vic to Cora Lynn, Vic	148 km
Mornington (Rosebud), Vic to Devon Meadows, Vic	50.6 km
Mornington (Rosebud), Vic to Cora Lynn, Vic	102 km
Mornington (Rosebud), Vic to Woori Yallock- Vic	111 km
Mornington (Rosebud), Vic to Werribee , Vic	120 km
Mornington (Rosebud), Vic to Trentham, Vic	186 km
Trentham, Vic to Manjimup, WA	3,483 km

One of the problems in defining the contemporary pathogen populations is that the samples themselves are genetically heterogeneous. Crute et al. (1980) pointed out that the collection of pathogens from the field may represent a mixture of different pathotypes of *P. brassicae*. One pathotype may dominate while the other pathotype(s) may occur at only a low frequency and this may lead to misleading predictions of virulence. The test is heavily dependent on all plants of an ECD set being infected uniformly; if they are not, the host may be mistakenly recorded as resistant or the result abandoned as unreliable (Kong Kaw Wa 2009). In such mixed collections, a less virulent pathotype may be masked by the dominance of a more virulent one, or a virulent pathotype present at a very low frequency may infect only a few plants, leading to a drop in the ECD score in pot trials (Dobson et al. 1983). To avoid this

possibility and decrease the possibility of a falsely low ECD code being recorded, Dobson (1982) recommended that all hosts that showed unreliable reactions should be re-inoculated with spores from the same infected ECD host, so that the frequency of that pathotype will greatly increase, to the point where susceptible reactions are produced (Dobson 1982). This, however, pre-supposes that a virulent population will overcome less virulent ones and that may not be the case.

Toxopeus et al. (1986) believed that even single galls would be composed of different pathotypes of *Plasmodiophora brassicae.* This may explain the emergence of new ECD codes with every study in Australia (Section 2.3, Table 2.3). Although collection 6 from cauliflower appeared to be a new pathotype in this study, previously it had been coded as 16/03/30 before storage at -20°C, suggesting that pathotypes are not stable or that the differences in environmental conditions between the two tests were sufficient to impact on disease expression. This was similar to the study by Jones et al. (1982b), in which replicate ECD tests with one collection of *P. brassicae* did not give consistent results. Jones et al (1982b) ascribed this to collections being heterogeneous and so to different parts of even a single gall containing different pathotypes. This suggests that pathotypes as tested in the ECD test are not stable through generations of plants and that new pathotypes are constantly being generated. This should be studied experimentally through several iterations in axenic conditions, as lack of stability would much reduce the value of generating an ECD code to compare pathotypes and to guide plant breeding for resistance. Any comparison of ECD codes depends on their reproducibility and that is not certain; this is discussed further in Section 2.4.2.

2.4.1.1 The reactions of *Brassica rapa* differential hosts (01–05)

The *B. rapa* host group (ECDs 01-05) had the greatest level of resistance of the three groups to the *P. brassicae* populations tested, in that all resulted in an ECD code of 16 and produced symptoms only in the most susceptible Chinese cabbage host ECD 05. This is similar to the results reported by Toxopeus et al. (1986). The high resistance of fodder turnip hosts ECD 01-05 in this group to Australian *P. brassicae* populations in general is probably a result of lack of adaptation of local populations to this crop, which is not commonly grown in most *Brassica* production regions in Australia. Logically, strong virulence to these *B. rapa* hosts is therefore unlikely to occur in Australian mainland pathogen collections and this probably explains the lack of infection of these hosts in the *B. rapa* group (Donald et al. 2006a). By contrast, fodder turnips are a common crop in the northern hemisphere and the pathotypes

there vary much more in virulence to *B. rapa* hosts (Donald et al. 2006a; Kong Kaw Wa 2009).

In the disease triangle, the occurrence of disease requires the coincidence of three factors, which are a susceptible host plant, a virulent pathogen and favourable conditions. From this standpoint, the infection of Chinese cabbage host ECD 05 is still a cause of concern, as Chinese cabbage is an important crop and favourable environmental conditions exist in Victoria for at least one genotype of *B. rapa*. Comparing the genetics of susceptible ECD 05 with the resistant ECDs 01-04 by microarrays and next-generation sequencing could potentially pinpoint which gene(s) are different and may be important for resistance.

This group is important in the search for sources of resistance and their later development. Some resistant turnip varieties developed in the Netherlands later became severely affected susceptible hosts (Tjallingii, 1960). Also, Tjallingii (1964) showed that several pathotypes (races) of *P. brassicae* could attack turnips and that they exhibited differential pathogenicity. Karling (1968) and Buczacki et al. (1975) found clubroot resistance in turnip (*B. rapa*). Matsumoto et al. (1998) identified a clubroot resistance locus, *CRa,* and its linkage markers in fodder turnip (ECD02). Suwabe et al. (2003) identified two clubroot resistance loci, namely *CRr1* and *CRr2*, which might occur on different regions or on different chromosomes. Hirai et al. (2004) discovered another dominant clubroot resistance locus (*CRr3)* in European turnip. Moreover, Piao et al. (2004) found another clubroot resistance locus (*CRb*) (in a hybrid Chinese cabbage cultivar) that was independent of *CRr1, CRr2* and *CRr3*. Additionally Sakamoto et al. (2008) described two clubroot resistance loci, *CRk* and *CRc* identified from an F2 population of Chinese cabbage.

2.4.1.2 The reactions of *Brassica napus* differential hosts (06-10)

On the basis of interaction between *B. napus* with these ten Australian populations, the *B. napus* group came in second place for resistance of host groups after *B. rapa*. This is because no population tested infected host ECD 09 (NZ resistant rape) as expected and most were unable to infect ECD 08 (rape) and ECD 10 (swede). Most susceptible reactions were restricted to only hosts ECD 06 and ECD 07 (rape). Population 10 from cauliflower collected at Manjimup did not infect any of this group of hosts. These results are similar to those recorded previously for Australian pathotypes (**Table 2.3**). Only 8/41 Australian pathotypes with values greater than 3 for the *B. napus* group of hosts were recorded by Donald et al. (2006a), while pathotypes worldwide produce denary values of 0-31 (Toxopeus et al. 1986).

In this context, Australian pathotypes are clearly atypical. Strelkov et al. (2006) tested different populations of *P. brassicae* from different areas in Alberta (Canada). The populations were quite different in their reactions to the *B. napus* host group; all pathogen populations produced very high levels of disease on *B. napus* var. *napus* (fodder rape) hosts ECD 06-ECD 09, with IDs ranging from 80-100%, which indicates that most *B. napus* hosts were highly susceptible. The reaction of the other *B. napus* host, ECD 10, ranged from highly to moderately resistant with IDs of 9-33%. The Canadian populations of *P. brassicae* in Alberta (Canada) appeared to be fairly homogenous, by contrast with the Australian populations (heterogeneous). The differences in the pathogenicity of the ten populations tested here suggest that they too are relatively heterogeneous. The similarities in the results could be attributed to the populations being collected close to one another as well as being exposed to a very limited host range of the vegetable brassicas.

Internationally, 70% of 834 *P. brassicae* collections investigated produced susceptible reactions on hosts ECD08 and ECD09 while 31% produced susceptible reactions on all the *B. napus* hosts (Kong Kaw Wa 2009). This contrasts markedly with Australia, where most Australian collections (80.5%) did not infect any of the *B. napus* hosts or caused a susceptible reaction on ECD06 and/or ECD 07 (Donald et al. 2006a) and similar results were noted in both this study and by Kong Kaw Wa (2009). These results show similarities to those from North America and contrast markedly with those from Western Europe (Dobson et al.1983). Both Australian and American populations of *P. brassicae* are probably derived from infections on *B. oleracea* or *B. napus* introduced with European settlement and may be slow to adapt to other species of *Brassica* because they are not widely grown in those countries. By contrast, all species have been cultivated widely in Europe for centuries and thus the virulence of the pathogen toward the *B. napus* group has had much longer to develop. It will be interesting to see if the pathogen develops more virulence as canola (*B. napus*) is grown more widely in Australia; from a small base in the 1980's, it is now grown widely in South-eastern and South-western Australia and exports are ~1.5 million tonnes per year (http://www.rirdc.gov.au/programs/established-rural-industries/pollination/canola.cfm). It would also be interesting to compare these results with pathotypes from colder countries such as New Zealand where *P. brassicae* was probably also introduced and a wider range of *B. rapa* and *B. napus* crops is cultivated.

These patterns of resistance in *B. napus* should be taken into account in the growing canola industry in Australia, as some resistant lines may be beneficial to *Brassica* plant breeders in

the future to develop sufficient resistance in crop cultivars (www.australianoilseeds.com.2011).

A high number of susceptible reactions for host ECD 07 was recorded, with the exception of population No.10, which was collected from Western Australia (WA). This host line (ECD 07) may be another widely susceptible host in addition to ECD 05 (Buczacki et al.1975, Crute et al. 1980; Toxopeus et al. 1986; Donald et al. 2006a) but is resistant to *P. brassicae* 'race 7' (Williams' differentials) (Jonsson 1981) and a collection from Rosemaund (UK) (Jonsson 1981; Jones et al. 1982b). Donald et al. (2006a) pointed out that races 6 and 7 were consistent with ECD race numbers 16/2/30, 16/0/30 and 16/2/31, 16/0/31 respectively, as reported in Victoria. Australian *P. brassicae* collections sent to the USA from Victoria were identified as race 6, and another collection from New South Wales was identified as races 3, 6 and 7 (Williams 1966). The lack of resistance in ECD 07 to Victorian isolates is therefore consistent with Victorian isolates belonging to race 6 rather than race 7.

Lastly, it seems clear that that Australian pathogen collections lack virulence towards *B. napus* hosts ECD 08, ECD 09 and virulence to ECD 10 is low, as reported in other studies (Donald et al. 2006a; Kong Kaw Wa 2009).

2.4.1.3 The reactions of *Brassica oleracea* differential hosts (11-15)

The *B. oleracea* hosts were the most susceptible to the pathogen collections tested, suggesting that all the collections were most virulent towards this group, for reasons already discussed. Three *B. oleracea* hosts (ECD 11, ECD 12 and ECD 15) showed differential reactions to the pathogen collections, whereas both ECD 13 and ECD 14 were uniformly susceptible. Although Dobson et al. (1983) recorded differentially susceptible reactions for all *B. oleracea* hosts without exception, this has not been borne out by more recent researchers, where similar uniformly susceptible reactions for ECD hosts 13 and 14 have been reported (Donald et al. 2006a; Kong Kaw Wa2009).

While susceptibility to the infection varied for ECD 11, ECD 12 and ECD 15, ECD 15 was more susceptible than the others, as symptoms occurred on all plants but did not always reach the specified level of DI>33, and so some were scored as ? instead of S, leading to a difference of 16 in denary value. The deficiency of clear susceptible reactions on the host ECD 15 was a concern as it meant that, for example, the codes 16/3/15 and 16/03/31 differed only in degree of infection of ECD 15. As the codes 16/3/31 and 16/2/31 were two of the most common in Australia (Donald et al. 2006a; Kong Kaw Wa 2009), the codes 16/3/15 and

16/3/31 were not as different as their scores might suggest – much depended on the final assessment of the symptoms on ECD 15 (kale). The differences may, however, be real, as kale is not grown widely in the areas of the pathogen collections, and the pathogen may be undergoing the same process in adapting to it as discussed earlier for *B. napus*.

Differences such as these are problematic in the ECD system because all scores are visual observations and personal estimations. This has occurred frequently; for instance Dobson et al. (1982) considered that all *B. oleracea* hosts (11-15) were universally susceptible hosts with one denary value, which was 31, while more recent studies using more exacting requirements for classification of the host as S have resulted in a range of values, most commonly from 12-31 (Donald et al. 2006a; Kong Kaw Wa 2009). These differences could be attributed to different pathotypes of *P. brassicae* being tested or the emergence of new pathotypes, but could also be attributed to small variations in the experimental conditions, e.g. temperature, moisture and age of inoculum that would affect the balance between host and pathogen in the disease triangle.

The differences in denary values and the appearance of new pathotypes indicate that *P. brassicae* collections are still aggressive and variable for this group. As this is the host group most widely grown in Australia, such variations must be taken into account in selecting resistance genes for plant breeding.

2.4.2 The ECD sets as a tool for studying variation in P. brassicae

The ECD system retains acceptance and satisfaction for many clubroot researchers, but there are still numerous problems associated with the differentiation of *P. brassicae* pathotypes based on the phenotypic responses of the ECD hosts.

The utility of the ECD test depends on its ability to pinpoint accurately the pathotypes present in different locations and new ones emerging, for the purposes of tracing sources of new infection and targeting pathotypes for breeding for resistance. These depend on the pathotype's stability and the ECD's reliability and there are problems with both aspects. In Victoria, populations collected in 1998 from cabbage at Werribee varied from an ECD code of 16/03/12 (Caroline Donald, DPI, pers. comm.) to 16/03/30 on re-examination after storage at -20°C (this study) and another population from cauliflower at the same locality had an ECD code of 16/03/31 (Donald et al. 2006a) while Kong Kaw Wa (2009) scored it as unreliable. Similar problems arise with populations from Trentham, Victoria, examined here

and by these previous researchers. Repeated longitudinal sampling and ECD testing would be required to investigate this, as there is a dearth of long-term data, partly because farmers stop growing brassicas as symptoms become more frequent and more damaging economically. Strelkov et al. (2006) have noted drift in pathotypes for canola in Canada and the same might reasonably be expected in Australia as canola becomes more widely grown.

There are some differences in ECD scores among Australian collections that may signify the presence of new pathotypes (Jones et al. 1982b; Donald et al. 2006a). Each study has produced new ECD codes new to Australia but only Donald et al. (2006a) have produced such high *B. napus* denary values for five collections, from the most comprehensive and the only national study in Australia. (**Table 2.6)**

There are several uncertainties in interpreting these outcomes. Firstly, the tests are on collections (populations), not isolates, and so may have more than one pathotype in the collection, the proportions of which could vary, leading to misleading proliferations of ECD codes. The presence of mixed pathotypes of *P. brassicae* within both soils and infected roots is well known (Jones et al. 1982b; Toxopeus et al.1986; Donald et al. 2006a). Most spore suspensions are prepared from fresh or frozen galls, not single spores. This increases the likelihood that the inocula used contain mixed pathotypes. This may explain the emergence of contradictory host reactions and indeterminate scores. It is preferable to use single spores, as stated by Kuginuki et al. (1999), but a minimum of 1000 spores is generally required for infection. Only a few single-spore isolates are available, where the original spore suspension has been applied to small seedlings in axenic soil-less media and then galls from successful infections used as subsequent inoculum to bulk up what may be a homogeneous inoculum (Jones et al. 1982b; Xue et al. 2008) and none was derived from Australian collections.

Table 2.6: ECD codes recorded in previous Australian studies and this study (similar colouring indicates the same ECD code).

Study by	Donald et al. (2006a)	Strelkov et al. (2006)	Bernard et al. (2006)	Strelkov et al. (2007)	Kong Kaw Wa (2009)	This study (2010)
ECD codes obtained	16/00/00	16/15/12	31/31/31	-/15/12	16/00/14	16/00/14
	16/00/29	16/15/00	19/31/31		16/02/00	16/02/12
	16/00/31	16/02/12	21/23/31		16/02/14	16/02/14
	16/01/31	16/00/14			16/02/15	16/02/15
	16/02/30				16/03/14	16/02/30
	16/02/31				16/03/15	16/02/31
	16/03/00					16/03/13
	16/03/08					16/03/14
	16/03/12					16/03/15
	16/03/13					16/03/30
	16/03/14					
	16/03/15					
	16/03/28					
	16/03/29					
	16/03/30					
	16/03/31					
	16/07/12					
	16/07/30					
	16/19/12					
	16/19/31					
	16/22/12					
	16/31/13					
	16/19/31					

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Also, there is the question of genetic heterogeneity in the host plants, which are grown from seed from obligately outcrossing parents. Jones et al (1982b) and Kuginuki et al. (1999) emphasised that, due to the genetic heterogeneity of the differential hosts, clubroot resistance genes are preserved by mass selection because of self-incompatibility. This would lead to differences in scores between replicate plants within treatments. This may explain what happened in this study, as 10% of the 150 total reactions recorded indeterminate resistance ($0 < DI < 33$) as a result of one or two susceptible reactions from a primarily resistant differential host. Jones et al. (1982b) stressed that a fundamental required for developing a differential series was that all hosts must be genetically uniform. Using tissue-cultured clones instead of seeds should remove the host's heterogeneity in replicate responses and methods are known for *B. oleracea* cultivars (Jones et al. 1982b; Toxopeus et al. 1986).

This is apart from the difficulties associated with minor variations in the environmental conditions used by each researcher. Adequately controlled growth space is one of the main problems with the ECD (Karling 1968; Buczacki et al.1975; Manzanares et al. 2000a) and if a large number of collections is under investigation, a large space is required. The system is also time-consuming because it takes several weeks to grow the plants sufficiently and in this time environmental factors such as light and humidity can change; these may affect results with successive experiments. There is also the problem of contamination, not only cross-contamination by *P. brassicae*, but also by other fungi and bacteria causing disease, and predation by insects and other invertebrates. In this study, there were ten collections and it was impossible to type all isolates simultaneously in the glasshouse and so multiple experiments had to be carried out. To avoid cross-contamination, the entire glasshouse had to be disinfected between batches, pots had to be sterilised in bleach and soil had to be autoclaved. Predation by caterpillars with collections 3 and 4 led to both trials having to be abandoned. Other researchers have reported similar problems (Karling 1968; Buczacki et al.1975; Manzanares et al. 2000a).

Even small changes in conditions under which the host plants are grown can affect final scores. Several environmental factors (temperature, photoperiod, soil moisture and lights) greatly influence many plants and some microorganisms, but so far the way in which these affect the virulence of *P. brassicae* resting spores or change the host's physiology so that it is less or more favourable to *P .brassicae* invasion and clubroot development are not well characterised (Colhoun 1961; Thuma et al. 1983; Donald et al. 2006a).

Stored pathogens of all types tend to lose viability and pathogenicity over time. This applies equally to *P. brassicae*. Methods for evaluating spore viability and therefore true inoculum concentration have been developed (Takahashi and Yamaguchi 1988) and should perhaps be used to equalise the viable zoospore inoculum pressure when conducting the ECD test. Ideally, pathogen inoculum should be prepared only from fresh clubroot collections, but this has not always been the case. A reduction in spore viability, pathogenicity or virulence since collection and storage at -20°C could explain the large number of intermediate classifications, especially for *B. oleracea* hosts, instead of susceptible (S).

Coding each pathotype's response to the ECD hosts based on susceptible reactions needs to be able to distinguish finely between symptoms, yet differences were often not clear-cut. Care had to be taken in interpreting the numerical pathotype designations and, while producing reliable scores from one individual, the degree of subjectivity required may make scoring unreliable between researchers, to the extent that intermediate (?) and susceptible (S) may vary.

An additional problem, given the multiplicity of use of the ECD method in many countries of the world, is that there are differences in arbitrary values used to determine the proportion of plants of a differential host that must be diseased for a pathogen collection to be identified as pathogenic to that host. This study followed Dobson et al. (1983), Donald et al. (2006a) and Kong Kaw Wa (2009) in requiring host reactions to have a DI equal to or greater than 33 to consider as reaction as susceptible. However, Toxopeus et al. (1986) reported only DIs equal to or greater than 80 as susceptible. These different methods for estimation can lead to different scores and this has the effect of limiting the interpretation of data from different sources.

2.4.3 Alternatives to the ECD series for studying pathogen variation

Even though the ECD test has many unresolved problems, it is currently the best tool available for defining the virulence of populations of *P. brassicae* in order to compare them with similar studies and to act as a guide in efforts to breed brassicas for resistance to *P. brassicae*. Because of the problems of accuracy and reliability, many clubroot researchers around the world believe there is an urgent need to reconstruct the ECD series (Dixon. 2001; Donald et al. 2006a). Japanese researchers have developed an alternative to the ECD series by using clubroot-resistant F1 hybrids and lines of *B. rapa* that led to an intelligible

classification of populations of *P. brassicae* in Japan (Kuginuki et al. 1999). Similarly, Strelkov et al. (2012) have developed a set of host plants specifically to examine the pathogenicity and virulence of collections to canola. This might improve accuracy and reliability for specific purposes but does not solve the other problems of time and space requirements or the problem that collections are not homogeneous entities and pathotypes may not be stable through generations of infected plants.

Molecular methods are commonly used to trace and type pathogens using PCR-based methods such as sequencing of the ITS region, RFLP, ISSR and RAPD. These methods have been used for at least 350 plant pathogens in the last 10 years alone (ISI search in Web of Knowledge at http://apps.webofknowledge.com). Such methods have potential to be applied to the study of *P. brassicae* and its hosts to study some of the questions raised here, such as diversity of the pathogen and its stability through successive generations of galls. It may even be possible to find molecular markers for different pathotypes or virulence and so eliminate the need to run ECD tests, as for Pipe et al. (1995), one of the first to find molecular markers for aggression, in *Ophiostoma ulmi*, the cause of Dutch elm disease.

The aim of Chapter 3 was therefore to use molecular methods to:

1. Investigate the genetic diversity of *P. brassicae* in galls from populations 1-10
2. Investigate if genotypes found were associated with pathotypes as defined by their ECD codes.

Chapter 3. Genetic diversity of some Australian populations of *Plasmodiophora brassicae*

3.1 Introduction

3.1.1 Importance of characterising *Plasmodiophora brassicae*

Clubroot disease caused by *P. brassicae* has become a serious problem in all brassica crop-growing areas in the world, including Australia. Internationally, there has been an increase in frequency of clubroot in many countries, e.g. Australia, Canada, Sweden, Germany, UK, France, Czechoslovakia, Japan, Korea and China. The estimated value of losses resulting from clubroot disease can be up to 1.6 t/ha (50% of yield) (Donald and Porter 2003b; Dixon, 2009a). Clubroot disease has been identified in 90 countries around the world where brassicas are popular crops, according to disease incidence surveys published by the Commonwealth Mycological Institute (CMI) and more recently by the European Plant Pathology Organisation (EPPO) (Dixon, 2009a) (**Table 3.1**). As the pathogen continues to spread, clubroot is now found throughout the world anywhere *Brassica* crops are grown and is the major source of disease-induced loss (Dixon, 2009a).

P. brassicae seems to have uniform morphology around the world. Traditional morphological observation is not sufficient to distinguish populations; consequently, more research is required to determine diversity in the pathogen populations (Buhariwalla et al. 1995a; Moller and Harling 1996).

As early as 1931, Honig reported that many field populations of *P. brassicae* varied in their pathogenicity. Thereafter different differential host systems were recommended to study pathogen variation in pathogenicity (Ayers 1957; Williams 1966; Buczacki et al. 1975; Some et al.1996) as discussed in Chapter 2.

The genetic diversity of *P. brassicae*, which may result in different pathotypes, can lead to difficulties in managing the pathogen (Manzanares-Dauleux el al. 2001; Fahling et al. 2003 and Osaki et al. 2008a). Moreover, the study of morphological characters and physiological tests are costly and time-consuming. Furthermore, these assays are not considered reliable because they are unstable as they vary with the environment (Setti et al. 2011). If a method

Table 3.1: List of countries with records of clubroot disease (based on data from the Commonwealth Mycological Institute (Dixon 2009a).

	Continent					
Africa	**Asia**		**Europe**		**Oceania**	**Western Hemisphere**
Angola: present	BruneiDarussalam: present	Malaysia: present	Austria: widespread	Netherlands: present	Australia: present	Argentina: present
South Africa: present	China: present	Peninsular Malaysia: present	Belarus: present	Norway: present	New Zealand: present	Brazil: present
	Anhui: present	Philippines: present	Belgium: present	Poland: present	Papua New Guinea: R.D	Rio Grande do Sul: present
	Fujian: present	Sri Lanka: present	Bulgaria: widespread	Portugal: present		Sao Paulo: present
	Gansu: present	Turkey: present	Channel Islands: present	Azores: present		Canada: widespread
	Guangdong: present	Nepal present *	Czechoslovakia (formerly)	Romania: present		Chile: present
	Guangxi: present		Denmark: widespread	Russian Federation: present		Guyana: present
	Hong Kong: present		Estonia: present	Russia(Europe): R.D		Mexico: present
	Hubei: present		Faeroe Islands: present	Siberia: present		Puerto Rico: present
	Hunan: present		Finland: present	Spain: present		Trinidad: present
	Jiangsu: present		France: present	Canary Islands: present		USA: widespread
	Jiangxi: present		Germany: widespread	Sweden: widespread		Alaska: present
	Taiwan: present		Greece: present	Switzerland: widespread		Hawaii: present
	Yunnan: present		Hungary: R.D	England: present		Venezuela: present
	Zhejiang: present		Iceland: present	Northern Ireland (UK): present		
	India: widespread		Ireland: widespread	Scotland: present		
	Israel: present		Italy: present	Wales: present		
	Japan: present		Sardinia: present	Yugoslavia: present		
	Korea, DPR: present		Latvia: present			
	Korea, Republic: present		Lithuania: present			

*Timila et al. (2008); R.D= restricted distribution.

could be found to identify molecular markers for virulence, it would largely replace the time-consuming and technically difficult European Clubroot Differential (ECD) system.

In Japan field populations of *P. brassicae* vary in pathogenicity and virulence (Tanaka and Ito 2013). Several pathotypes (races) have been reported in Japan as well as in other countries such as France and Canada using numerous differential systems. As a result, it is likely that each field population is heterogeneous, consisting of multiple genotypes. Moreover, this diversity in the pathogen might be the reason for the failure of resistance in some clubroot-resistant varieties such as radish (*Raphanus sativus* subsp. *longipinnatus*) and could disrupt efforts to develop clubroot-resistant (CR) varieties.

Many workers have sought and used molecular markers to address these limitations and analyse the relationship between genetic variation and pathogenicity in populations and single-spore isolates of *P. brassicae* (Buhariwalla et al. 1995a; Moller and Harling 1996; Yano et al. 1997; Ito et al. 1999b; Manzanares-Dauleux et al. 2001; Fahling et al. 2003; Osaki et al. 2008a).

Investigation of characters such as aggressiveness and virulence, those most often used for studies of variation in *P. brassicae,* have provided clear differentiation of races and pathotypes, and their diversity and distribution, yet do not allow genotypic and hence evolutionary relationships among pathotypes to be distinguished (Lopez et al. 2003; Schaad et al. 2003). DNA marker technologies have been widely used in plant pathology, both to devise specific markers to trace pathogens and to study their diversity (Setti et al. 2011). Using such molecular markers can offer fast, accurate identification along with more rapid means to detect of plant pathogens in contaminated specimens (Bridge et al. 2003; Schaad et al. 2003; Setti et al. 2011). Many perplexing puzzles and questions can be answered, for instance the sources of inoculum and changes in pathogen population diversity.

3.1.2 Molecular characterisation of *Plasmodiophora brassicae*

Traditional methods such as disease morphology traits have been used to measure clubroot pathogenicity and variation, but cannot detect genetic variation without some morphological or physiological expression of the genes. Molecular analysis has been conducted for several years as a reliable, powerful and productive tool to highlight sources of inoculum and show genetic diversity of pathogens. The use of polymerase chain reaction (PCR)–based methods in plant pathology has increased massively, with several DNA marker systems being utilised

(Setti et al. 2011). Successful chemical and biological control of pathogen populations is strongly linked to understanding the level of pathogen diversity (Buhariwalla et al. 199a; Manzanares-Dauleux el al. 2001).

Several PCR-based molecular methods are widely used, such as specific primers based on the nuclear ribosomal Internal Transcribed Spacer (ITS) sequences to trace the pathogen, and Randomly Amplified Polymorphic DNA (RAPD) and microsatellite-based simple sequence repeats (SSR) to study diversity (Milgroom 1997). Faggian et al. (1999) showed that specific primers targeted to the high-copy ITS region could be used to trace and quantify *P. brassicae* in the environment. This method of detection of *P. brassicae* could trace sources of contamination but could not be used to study pathogenic and genetic variation because of the uniform nature of the ITS sequence on which the primers are based (Faggian 2002).

PCR amplification of repetitive DNA fragments in RAPD and SSR from *P. brassicae* DNA revealed polymorphism in the target genome and hence characterisation of genetic variation (Buhariwalla et al. 1995a). RAPD and microsatellite primers have been used to differentiate between populations of *P. brassicae* outside Australia (Buhariwalla et al. 1995a; Moller and Harling 1996; Manzanares-Dauleux el al. 2001; Rosa et al. 2010).

Feng et al. (2013) analysed the genes that are expressed during clubroot formation. They used suppression subtractive hybridization (SSH) and expressed sequence tag (EST) analysis; a cDNA library constructed by SSH consisted of 797 clones that represented 439 unigenes. Thirty-two of these genes were demonstrated to be of a *P. brassicae* origin, and of these, 24 had not been previously reported.

3.1.2.1 Specific PCR primers for *P. brassicae*

The detection of soil-borne pathogens has posed formidable problems because of their irregular distribution in soil that may be mixed with saprophytes and other microorganisms. Finding such pathogens is slow because it has depended on baiting with susceptible plants, which is labour-intensive and has sometimes yielded inconclusive results. By contrast, molecular techniques are able to provide precise, reliable and reproducible results rapidly, facilitating early disease management decisions.

Faggian et al. (1999) showed that a nested PCR assay using primers targeted to ribosomal DNA genes and ITS regions could detect *P. brassicae* in soil and other environmental

samples. The nested PCR assay amplified a final product of 620 bp from an initial product of 1100 bp. The assay had detection limits of 0.1 fg (10^{-16}g) for pure template and detected as low as 1000 spores/g of potting mix. Ito et al. (1999a) also used a nested PCR of a different part of the DNA to amplify a 398-bp product internal to an initial 1457 bp fragment in a single-tube nested PCR (STN-PCR) format. It was claimed that it is possible to detect even a single resting spore per gram of soil. Both primers have ready use in detecting contamination and tracing sources to the origins, as well as allowing confirmation that DNA samples do contain *P. brassicae* DNA capable of reacting in PCR and so testing of negative results with other primers.

3.1.2.2 RAPD primers

Williams et al. (1990) and Welsh and McClelland (1990) are the early researchers who simultaneously developed the use of RAPD (random amplified polymorphic DNA). The technique amplifies the template DNA without requiring sequence information on the target (Setti et al. 2011)

The RAPD technique depends on the use of small primers, generally 5-15 bp in length. In this reaction, oligonucleotide primers of random sequence operate in both forward and reverse directions, annealing to the genomic DNA at constant low annealing thermocyclic temperatures (Williams et al., 1993). In the thermal cycle of RAPD reactions, when an appropriate annealing temperature is reached, short oligonucleotide primers of random sequence start to bind to several priming sites on the complementary sequences in the template genomic DNA and produce discrete DNA products if these priming sites are within an amplifiable distance of each other, leading to a fingerprint of inter-binding site fragments of variable length (**Fig. 3.1**). The resulting PCR products are resolved on agarose gel and visualised with ethidium bromide or fluorescently labelled nucleotides. The resulting products appear as bands on the gel and are scored as present or absent for each individual, producing binary data for statistical interpretation (Bardakci 2001; Setti et al. 2011).

RAPD has many advantages. It requires no prior knowledge of the genome and so the same primers can be used for any organism. Moreover, the technique is simple to set up and fast to assay, and hence a very large number of markers can be screened in very short period. Furthermore, as this technique is PCR-based, it is very sensitive and only very small quantities of DNA (1-20 ng) are needed (Williams et al. 1993; Setti et al. 2011).

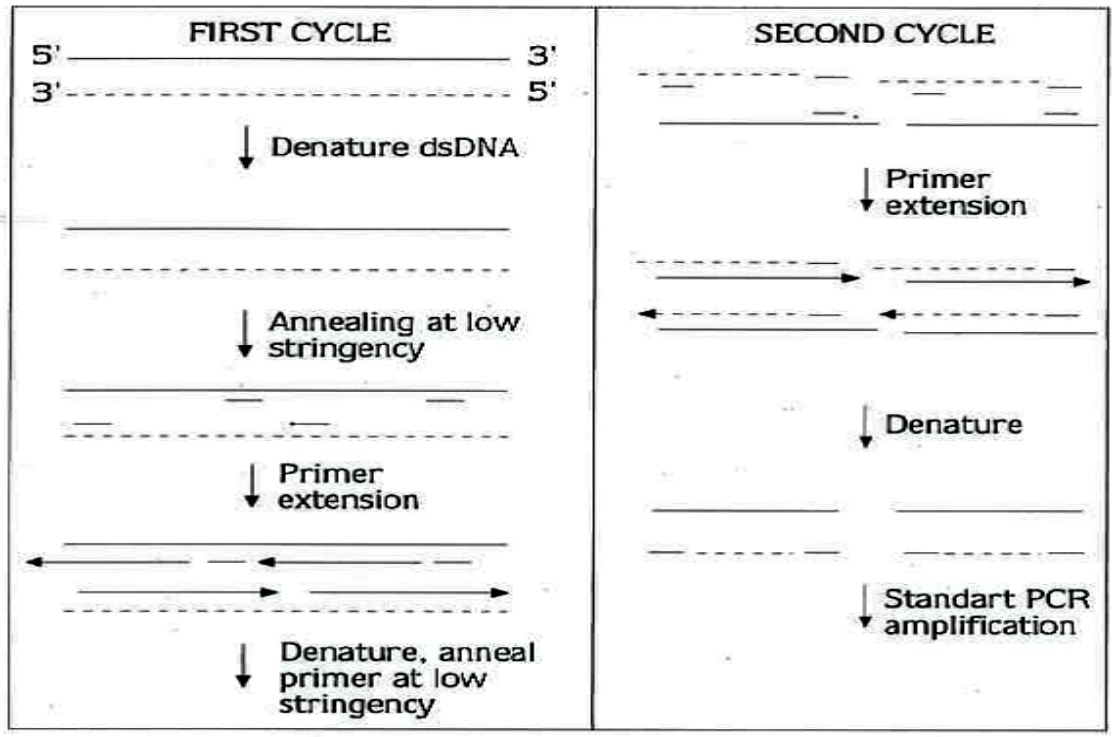

Figure 3.1: Schematic diagram of RAPD reactions for two loci. (Derived from Welsh and McClelland (1994) as cited by Setti et al. 2011).

There are, however, some drawbacks of RAPD primers. The resulting patterns of bands are very sensitive to variation of the experimental conditions and DNA quality. Also, with some samples there is low reproducibility, which makes RAPD markers unsuitable for comparison between laboratories (and sometimes, it is claimed, within laboratories) for similar species; to avoid this issue, it is preferable to repeat the assay 3-4 times. Also, it is claimed not to be acceptable for diploid or polyploid fungi such as the basidiomycetes and ascomycetes as the alleles cannot be differentiated (Setti et al. 2011). However, RAPD was used to show the size of a single colony of *Armillaria mellea* and to show that RAPD markers segregated in the spores (Otieno et al. 2003). Moreover, Caldeira et al. (2009) characterised *Amanita ponderosa* by using RAPD amplicons as biomarkers.

3.1.2.3 Microsatellite primers (SSR)

In SSR analysis, the motifs that are targeted are repeated several times at various points of the DNA in all organisms, both prokaryotes and eukaryotes. Litt and Luty (1989) created the term microsatellites, also referred to as simple sequence repeats (SSRs) and short tandem repeats (STRs), with a base motif of 1-10 bp, that are repeated in varying numbers of repeats scattered around the genome. The targeting of tandem repeats in PCR results in the primers

annealing to both strands of the DNA template at several points, similar to RAPD. This results in the production of multiple products that form banding patterns that are frequently polymorphic, resulting in this technique being referred to as simple sequence length polymorphism (SSLP) (Semagn et al. 2006; Setti et al. 2011). The repeated sequences are usually simple, comprising 1-6 bp (usually 2-4 bp), and they are present in both the coding and non-coding regions. The simplest common single repeat is $(CA)_n$ (Groppe et al., 1995; Morgante et al., 2002). Microsatellite markers are believed to be relatively stable, with a change rate of about 10^{-2} to 10^{-4} per generation (Setti et al. 2011).

The PCR primers for microsatellites are specific oligonucleotides designed by using information concerning the repeats of the flanking regions. Hence, the forward and reverse primers are used to anneal at the 5' and 3' end of the template DNA respectively. PCR products are usually separated on polyacrylamide or agarose gel and visualized by staining with ethidium bromide or by autoradiography (Matsuoka et al. 2002).

The advantages of using microsatellites are their polymorphism, abundance and distribution throughout the genome. This allows the distances between SSRs to be used to see polymorphism that reflects the enormous extent of allelic diversity and has made the microsatellites one of the most popular markers. Also this technique is PCR-based and so only a small quantity of DNA template is required. Furthermore, the reproducibility of this technique is high and it can be used effectively by different research laboratories to produce consistent data.

The production of DNA fingerprints by using polymorphisms as a basis can be performed by a number of routes:

(i) PCR (MP-PCR - microsatellite-primed PCR), in which direct amplification of microsatellite regions uses oligonucleotide primers designed to bind to microsatellite sequences, e.g. $(GACA)_4$ and $(GTG)_5$.

(ii) These same oligonucleotides can be used as hybridisation probes to reveal restriction fragment length polymorphism at multiple loci after digestion of DNA (Weising et al. 1995; Buscot et al. 1996)

(iii) Beckmann and Soller (1990) suggested a method termed sequence-tagged microsatellite site (STMS), which means microsatellites can be implemented as monolocus, codominant markers by converting individual microsatellite loci into

PCR-based markers by designing primers from unique sequences flanking the microsatellite.

3.1.2.4 RAPD vs SSR

For studying genetic diversity in *P. brassicae*, both RAPD and SSR (SG-PCR and MP-PCR) have the potential to develop diagnostic markers that could allow for easy genotyping of the pathogen populations, ideally aligning with virulence and ECD tests. Their properties are very similar but RAPD typically produces more polymorphic loci than SSR (**Table 3.2**). Reproducibility is reported as greater in SSR than RAPD, the main problem being some faint bands that are not consistent in RAPD. This means that SSR is held to be more accurate but less discriminatory than RAPD.

Table 3.2: Comparison of RAPD and SSR used in genetic diversity studies.

Features	RAPD	SSR	Reference
Major application	Gene tagging/ Genetic diversity	Genetic diversity	Buhariwalla et al. (1995a); Bardakci (2001), Manzanares-Dauleux et al. (2001)
Quantity of DNA required	0.02 (low)	0.05 (low)	(Setti 2011)
Technical requirement	Low	Medium	
Degree of polymorphism	Medium	Medium	
Number of polymorphic loci analysed	1.5-50	1.0-3.0	
PCR-based	Yes	Yes	
Reproducibility	Medium*	High	
Prior information needed	No	Yes/No	
Ease of use and development	Easy	Easy	
Cost per analysis	Low	Low	
Automation	Moderate	High	
Accuracy	Variable*	High	
Radioisotope detection	No	No	

* Special care is needed to exclude DNA from other sources of DNA from being amplified (Bardakci, 2001).

3.1.3 Genetic diversity of *Plasmodiophora brassicae*

Molecular analysis utilising the polymerase chain reaction (PCR) has now been conducted for several years as a reliable, powerful and productive tool for highlighting genetic diversity of *P. brassicae*, but not in Australian populations. PCR amplification of DNA using RAPD or SSR primers often results in polymorphism in the products and hence characterisation of genetic variation (Buhariwalla et al. 1995a; Moller and Harling 1996; Manzanares-Dauleux et al. 2001).

Several researchers have tried to analyse the relationship between the diversity in pathogenicity and DNA polymorphism in the populations of *P. brassicae* (Buhariwalla et al. 1995a; Moller and Harling 1996; Yano et al. 1997; Manzanares-Dauleux et al. 2001; Fahling et al. 2003; Osaki et al. 2008a; Osaki et al. 2008b; Rosa et al. 2010).

Buhariwalla et al. (1995a) were the first to investigate genetic diversity in *P. brassicae*, using a mixture of primers: RAPD and microsatellite (SG and MP) to investigate populations from northern Europe (Sweden, UK, France and Germany). This distinguished nine single-spore isolates and populations of field-collected *P. brassicae* through the amplification of reproducible banding patterns. The most successful primers were the SG primers HKB17/9, HKB 17/33 and HKB23/52; and the RAPD primers (OPA-3, OPA-09 and OPA-20). Banding patterns were not clear and could not be reproduced consistently. This lack of reproducibility and clarity was ascribed to potential contamination of resting spores of *P. brassicae* by microbes or host DNA during the extraction process, or by host DNA taken up by *P. brassicae* during the infection process.

Moller and Harling (1996) disagreed, showing that the amplification of *P. brassicae* from three single-spore isolates from UK and Germany with 23/40 Operon RAPD primers resulted in simple products in standardised conditions. Three of 23 Operon primers resulted in profiles specific to the isolates and one profile (from OPA-07) corresponded to the pathotype classification. They pointed out, however, that they had examined only three isolates and that many more were needed.

Some et al. (1996) showed variation in virulence from 20 field collections of *P. brassicae* from France. These authors confirmed that the single spore and field isolates had a unique multilocus molecular genotype population of *P. brassicae*, and were highly heterogeneous for both virulence and DNA pattern. Manzanares-Dauleux et al. (2001) emphasised the

importance of choosing breeding tactics to develop durable clubroot resistance because of the ample genetic differences occurring in *P. brassicae* populations. This was followed (Manzanares-Dauleux et al. 2001) by a study of the genetic diversity of nine field-collected populations and 37 single-spore isolates from France using 19 RAPD primers and nine SG primers, which showed that populations and isolates were composed of 11 weak clades with low bootstrap values. Only two RAPD primers were related to one of the pathotype codes. There was no clear relationship between the molecular analysis and the pathogenicity tests, suggesting that these are unlikely to be classified as a heterogeneous group of isolates according to pathotype, host or geographical origins.

This study was followed by Yano et al. (1997), who demonstrated that RAPD analysis could clearly differentiate some of Williams' races, but did not differentiate virulent populations found in Japan. Yano et al. (1997) found that field populations of *P. brassicae* produced various RAPD patterns corresponding to the arbitrary primers used. They indicated that out of 16 populations tested only two (YAYH and SS) showed unique RAPD patterns and were clearly distinguishable from the 14 other populations. These two populations (YAYH and SS) were previously classified as Williams' race 9. Both varied considerably in virulence and aggressiveness to some crucifers and common clubroot-susceptible hosts of other populations. This supports work carried out by Kuginuki et al. (1999) and Hatakeyama et al. (2004) that reported the presence of four pathotypes on CR cultivars in *P. brassicae* populations from the same region.

A Japanese study conducted by Osaki et al. (2008a) found that 17 populations of *P. brassicae* in Japan showed a comparatively high level of genetic diversity, and some of the population pathotypes were considered to be extremely heterogeneous. Additionally, the population was thought probably to be genetically polyphyletic, and these populations could occur during plural evolution processes. Also, no evidence for any pattern of geographical distribution of the pathotypes could be determined.

3.1.4 Problems with the use of molecular tools with *Plasmodiophora brassicae*

Several studies have attempted to understand the molecular and cellular biology and biochemistry of *P. brassicae*; however so far there is still some ambiguity in this aspect despite the economic importance of the pathogen and the number of studies that have been conducted to find its causation (Siemens et al. 2009a). The need to increase our knowledge of *P. brassicae* genetic variation is clear, but as an obligate pathogen, there are problems with the techniques used to study molecular mechanisms of pathogenesis (Hwang et al. 2012a).

Bulman et al. (2006) located the pathogen genes in roots of the model organism *Arabidopsis thaliana* by inhibiting hybridisation between RNA from *P. brassicae*-infected and uninfected *Arabidopsis* tissue as well as screening full-length cDNA clones from infected tissue by using oligo-capping procedures. They identified 76 new gene sequences of *P. brassicae*, the majority of which were extended to complete length at the 5' end by the use of RACE amplification.

3.1.4.1 Contamination with foreign DNA

Siemens et al. (2009a) stated that one of the most important difficulties when working on the genetic level is to obtain pure genetic material (DNA) free from contamination from host plant materials or other organisms. *P. brassicae* DNA is extracted from resting spores of the pathogen. While these preparations are highly enriched with plasmodiophorid material, unspecific microbial contaminations are present and poor yields of RNA are obtained from the spores. This has so far prevented simple expressed sequence tags (EST) being sequenced from this material.

3.1.4.1.1 Host plant DNA

One of the main problems is contamination of the *P. brassicae* DNA with DNA from the host plant. Preparations of spores from within galls have been found to contain plant DNA (Faggian 2002). Therefore it is imperative in an assay using non-specific primers to assay the host plant DNA from which the galls were removed in order to prevent false conclusions.

3.1.4.1.2 Soil microorganisms

As clubroot galls are in contact with the soil, the DNA of soil microorganisms could be extracted along with that of *P. brassicae*. Although peeling the outside of the galls (Manzanares-Dauleux et al. 2001) assists in removing surface contaminants, contaminating DNA may amplify in non-specific techniques like RAPD- and microsatellite-primed PCR.

Contaminating fungal DNA from common soil-borne fungi (e.g. *Penicillium, Exophiala*) may be detected by using the fungal-specific primer ITS-1F (Gardes and Bruns 1993) along with the universal primer ITS-4 (White et al. 1990). Contaminating DNA from soil bacteria (e.g. *Flavobacteriumsp sp.; Rhodococcus sp.* and *Variovorax sp.)* can be detected by using two universal bacterial primers – the forward primer fD1 and the reverse primer rP2 for 16s rDNA (Weisburg et al. 1991).

3.1.4.1.3 Genetic heterogeneity

Jones et al. (1982a,b), Fahling et al. (2003) and Siemens et al. (2009a) have shown that populations of *P. brassicae* exist as mixtures of pathotypes in the soil and even in single clubbed roots, while Xue et al. (2008) also conducted work with a large collection of single-spore isolates. Most *P. brassicae* samples used for experimentation are likely to be heterogeneous mixtures of genotypes and so even DNA extracts from different galls on the same root could be characterised as different genotypes. The requirement to obtain consistent results has been the impetus behind the generation of single-spore isolates, where a single spore has produced a single gall and so theoretically should be genetically homogenous (Faggian 2002; Kong Kaw Wa 2009). This requires the initial spore to be uninucleate and haploid or homozygous, an assumption not necessarily warranted.

3.1.5 Pathogenicity and virulence in *Plasmodiophora brassicae*

The relationship between genetic and pathogenic diversity within *P. brassicae* has been studied by researchers for many years (Buhariwalla. et al., 1995a; Some et al., 1996 ; Moller and Harling 1996; Yano et al. 1997; Manzanares-Dauleux. et al. 2001; Fahling et al. 2003; Osaki et al. 2008a).

Molecular markers can distinguish more genotypes than the ECD types derived from using differential hosts. Thus some field populations can have a very complex mix of genotypes but show no or little variation in ECD pathotype (Donald et al. 2006a; Kong Kaw Wa 2009). This is also seen in the diversity for virulence (and by implication genetic diversity) among single-spore isolates even from a single field-collected clubroot sample. This is complicated by the demonstration that in some cases there was selection for specific pathotypes during the single-spore extraction process (Crute.et al. 1980; Linnasalmi and Toiviainen 1991; Buhariwalla et al. 1995a; Voorrips 1995; Moller and Harling 1996; Kuginuki et al. 1999; Manzanares-Dauleux et al. 2001; Heo et al. 2009; Rosa et al. 2010).

Field populations of *P. brassicae* are believed to consist of mixtures of genotypes, and thus may carry many different non-virulence and virulence genes. This large genetic variation in field populations of *P. brassicae* may enable the pathogen to combat any resistance developed in the plant hosts and presents a huge challenge for the development of clubroot-resistant varieties. Several researchers attempting to understand the structure of field populations of *P. brassicae* have concluded that different ECD codes detected in the same

field population are probably due to an uneven distribution of genotypes in the spore populations present in the galls used to prepare the spore suspension to inoculate ECD plant hosts, which must be a reflection of the genetic diversity (Crute et al. 1980; Scholze et al. 2002; Donald et al. 2006a). Suitable molecular methods for assessing the genetic diversity within and between population and field isolates are vital (Buhariwalla et al., 1995a).

3.1.6 Aims

The aims of this chapter were to assess genetic variation among the ten field populations of *P. brassicae* used in Chapter 2 by PCR with two sets of primers:

1) RAPDs (three sets of 20 arbitrary primers – Operon OPA, OPB and OPM)
2) microsatellites (HKB 17/9, HKB 17/33, HKB 23/52, $(GACA)_4$ and $(GTG)_5$).

The specific objectives were to investigate genetic variation in these Australian populations of *P. brassicae* by:

a- assessing the usefulness of each primer in detecting genetic polymorphism in populations
b- analysing the genetic polymorphism in populations
c- assessing the relationship, if any, between genotype and ECD pathotype.

3.2 Materials and Methods

3.2.1 Pathogen and other materials

3.2.1.1 *Plasmodiophora brassicae* populations

The ten Australian *P. brassicae* populations analysed genetically were the same as those in Chapter 2 for ECD trials. DNA was extracted from the original galls as explained below and tested with ITS, specific, RAPD and microsatellite primers.

3.2.1.2 Common clubroot host plants

Four common host plants for clubroot disease (broccoli, Chinese cabbage, cabbage and cauliflower) were used (**Table 3.3**). Twenty seeds of each species were surface-sterilised, treated at 4ºC, planted and grown in a glasshouse as in Chapter 2. Each species had five pots with four seeds per pot. All plants were watered every 2 days and grown in a glasshouse as in Chapter 2 for 10 weeks.

Table 3.3: Common clubroot host plants used for DNA extraction

Common name	**Scientific name**	**Cultivar**	**Source**
Broccoli	*Brassica oleracea* L. var. *italica*	Calabrese broccoli	Syngenta Seeds Pty Ltd
Cabbage	*Brassica oleracea* L. var. *capitata*	Green cabbage	Syngenta Seeds Pty Ltd
Chinese cabbage	*Brassica rapa* L. var. *chinensis*	Pak choi	Syngenta Seeds Pty Ltd
Cauliflower	*Brassica oleracea* L. var. *botrytis*	White cauliflower	Syngenta Seeds Pty Ltd

3.2.1.3 Contaminant fungi

Two fungi identified from sequencing of ITS-1F/ITS-4 products were retrieved from the Mycology culture collection at the School of Applied Sciences, RMIT University: *Exophiala dermatitidis* (Kano) de Hoog (RMIT No. 171) and *Penicillium chrysogenum* Thom (RMIT

No. 547). These were subcultured on to potato dextrose agar (PDA – Oxoid) and grown at 26ºC to check purity. Cultures of *Escherichia coli* were also retrieved.

3.2.2 DNA extraction

3.2.2.1 Clubroot galls

In order to minimise host plant DNA, the procedure of Manzanares-Dauleux et al. (2001) was followed. Galls were washed carefully and the outer layer of each gall for each sample peeled off. Each peeled gall sample was dispersed in 100 mM $MgCl_2$ in 200 mM Tris buffer, pH 7.4 and treated with DNase I (Sigma, D-4263) (30 µg mL^{-1}) for 3 hours at 37ºC to eliminate host DNA. The suspension was centrifuged at 2500 g for 5 min and the pellet was digested in 5 mM EDTA and 0.5% SDS in 10 mM Tris buffer, pH 7.8, containing 20 mg mL^{-1} proteinase K for 30 min at 37ºC. After centrifugation as before, the final pellet was stored at -20ºC (Manzanares-Dauleux et al. 2001). From each sample, DNA was extracted using a Qiagen DNeasy Plant Mini-kit using the manufacturer's protocol. Each sample (100 mg) was ground in liquid nitrogen in a mortar and pestle. The resulting powder was transferred to a 2 mL Eppendorf microcentrifuge tube containing 400 µL of buffer AP1 and 4 µL of RNaseA stock solution, vortexed and incubated for 10 min at 65°C, with the tubes being inverted and vortexed two-three times during incubation. Then 130 µl of buffer AP2 was added to the lysate and incubated for 5 min on ice. The lysate was centrifuged for 5 min at maximum speed (20000 x g) which was then followed by applying the lysate to the QIAshredder spin column and centrifugation for 2 min at (20000 x g). The flow through fraction was transferred to a new tube without disturbing the cell-debris pellet. A 1.5 x volume of buffer AP3/E was added to the tube with the flow-through fraction and mixed by pipetting in order to precipitate the DNA. This mixture was applied to a DNeasy mini spin column with a 2 mL collection tube and centrifuged for 1 min at 6000 x g. The flow-through was discarded and the same step was repeated with the remaining sample. To wash DNA, the DNeasy mini column was placed into a new collection tube and 500 µL of buffer AW was added to the DNeasy mini column and centrifuged at 6000 x g. This step was repeated after discarding the flow-through. The DNeasy mini column was placed in a 1.5 mL microcentrifuge tube and 100 µL of pre-heated buffer AE (to elute the DNA) was added directly to the DNeasy membrane, incubated for 5 min at room temperature and then centrifuged at 6000 x g for 1 min. The DNA extract was stored at -20ºC.

3.2.2.2 Common clubroot host plants

Roots were harvested from 10-week-old plants, washed and dried and then DNA was extracted from 100 mg samples of fine roots of each species using a Qiagen DNeasy Plant Mini-kit using the manufacturer's protocol as for galls.

3.2.2.3 Contaminant fungi and bacteria

Yeasts were scraped from cultures of *E. dermatidis* and hyphae of *P. chrysogenum* were picked from cultures on PDA, avoiding agar. Aliquots of 100 mg of each fungus were weighed and DNA was extracted using a Qiagen DNeasy Plant mini-kit following the manufacturer's protocol as for galls. Growths of *E. coli* were scraped from overnight culture and extracted in the same way.

3.2.2.4 Determination of purity and quantity of genomic DNA

The purity and quantity of genomic DNA was determined spectrophotometrically and by electrophoresis in 1% agarose gel in TBE buffer (54 g Tris-base , 27.5 g boric acid, 20 ml of 0.5 M EDTA, pH 8.0) against known concentrations of the molecular weight marker GeneRuler (Fermentas).

3.2.3 Polymerase chain reaction (PCR)

DNA was amplified by PCR (polymerase chain reaction) using several different sets of primers for different purposes:

- to check that the DNA was capable of reacting in PCR, the universal primer pair ITS-1 and ITS-4 to the ITS (internal transcribed spacer) region of nuclear ribosomal DNA (White et al. 1990; Grades and Bruns 1993) (**Table 3.4**).
- to check that the samples contained DNA from *P. brassicae* that was capable of reacting in PCR, the specific primers pair PbITS1, PbITS2 followed by the nested primer pair PbITS6 and PbITS7 (Faggian et al., 1999) (**Table 3.4**)
- to detect contaminating fungi, the primer pair ITS-1F and ITS-4 (Gardes and Bruns 1993)
- to detect contaminating bacteria, the primer pair fD1 and rP2 (Weisburg et al. 1991) (Table **3.2**)
- to detect genetic polymorphism in the DNA from the galls, two types of primers:

- RAPD (randomly amplified polymorphic DNA) primers Operon sets OPA, OPB and OPM, each consisting of 20 10-mer primers
- Microsatellite (ISSR – inter-simple sequence repeat) primers used previously with *P. brassicae* (Buhariwalla et al. 1995a; Faggian 2002).

3.2.3.1 Universal primers (ITS-1 and ITS-4)

The sequences and expected products sizes for the universal primer pair ITS1 and ITS4 are shown in **Table 3.2**. The standard PCR protocol used was 9.5 µL of PCR water (nuclease-free water -Promega), 1 µL of each primer, 12.5 µL of GoTaq Green Master mix (Promega) and 1 µL of extracted DNA of each sample in a total reaction volume of 25 µl. PCR reactions were performed in a GenAmp 2400 thermocycler (PerkinElmer, CA). The following reaction cycle was kept standard for all the experiments. Initial denaturation at 94ºC for 10 min, 35 cycles of denaturation at 94ºC for 30 s, annealing at 50ºC for 30 s, extension at 72ºC for 1 min, and final extension for 10 min at 72ºC with a holding temperature of 4°C. The PCR products were electrophoresed on a 1.5% agarose gel in TBE (54 g Tris-base, 27.5 g boric acid, 20 mL of 0.5 M EDTA, pH 8.0) for 60 min at 120 V in a Bio-Rad electrophoresis apparatus and visualised by staining with 0.5 µg/mL ethidium bromide, destaining and recording images with a Gel-Doc 2000 imaging system (Bio-Rad). All PCR experiments included at least one negative control which contained nuclease-free water (Promega) or MilliQ water. Each test was repeated at least three times.

3.2.3.2 Specific primers (PbITS1/PbITS2) and nested primers (PbITS6/PbITS7)

The specific primer pair PbITS1/PbITS2 was tested to generate PCR product from *P. brassicae* in the galls (**Table 3.4**) along with a set of nested primers (PbITS6/PbITS7) for greater sensitivity. Primers PbITS1 and PbITS6 are both complementary to regions within the 18S rDNA fragment, whereas PbITS2 and PbITS7 are complementary to regions with ITS2 and ITS1 respectively (**Fig. 3.2**) (Faggian et al. 1999; Faggian 2002). PCR compositions, conditions and cycles were as for universal primers above in the initial cycles with PbITS1/PbITS2. For the subsequent nested PCR with PbITS6/PbITS7, 1 µL of the PCR product from the PbITS1/PbITS2 reaction was used instead of genomic DNA.

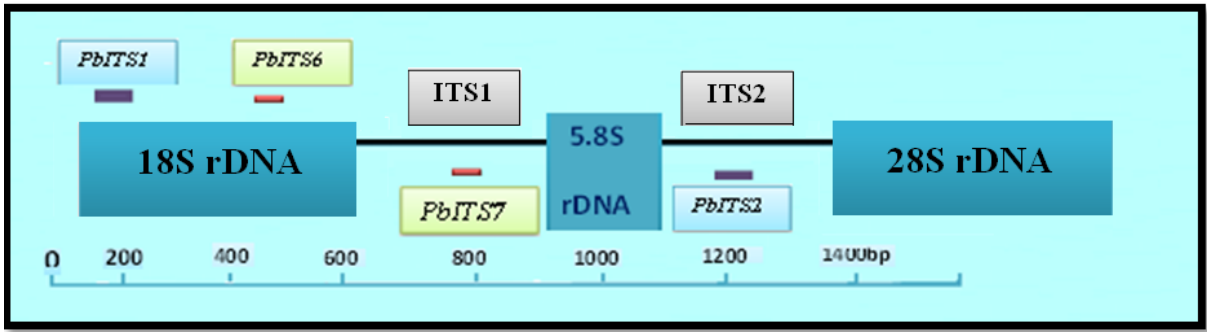

Figure 3.2: Diagrammatic illustration of the ribosomal DNA repeat of *Plasmodiophora brassicae* showing the approximate locations of the specific primers PbITS1 and PbITS2 and the nested primers PbITS6 and PbITS7 (Faggian et al. 1999).

Table 3.4: Characteristics of primers used in the various PCR reactions

Primer	**Sequence 5'→3'**	**Expected product size (bp)**	**Reference**
ITS-1	TCCGTAGGTGAACCTGCGG	650-700 bp	White et al. (1990)
ITS-4	TCCTCCGCTTATTGATATGC		
PbITS1	ACTTGCATCGATTACGTCCC	1086-1100 bp*	Faggian et al. (1999)
PbITS2	GGCATTCTCGAGGGTATCAA		
PbITS6	CAACGAGTCAGCTTGAATGC	507-620 bp*	Faggian et al. (1999)
PbITS7	TGTTTCGGCTAGGATGGTTC		
ITS-1F	CTTGGTCATTTAGAGGAAGTAA	550-700 bp	Gardes and Bruns (1993)
fD1	ccgaattcgtcgacaacAGAGTTGATCCTGGCTCAG	~1400bp	Weisburg et al. (1991)
rP2	cccgggatccaagcttACGGTTACCTTGTTACGACTT		

*Predicted from sequence of *P. brassicae*, accession no Y12831 (Ward and Adams 1998)

3.2.3.3 Fungi-specific primer ITS-1F with universal primer ITS-1

PCR compositions, conditions and cycles were as for universal primers above, apart from the primer ITS-1F (Gardes and Bruns 1993) are being used instead of ITS-1.

3.2.3.4 Bacteria-specific primers fD1 and rP2

Approximately 1 µg of genomic DNA of bacteria (*Escherichia coli*) (as a positive control) and galls were amplified in a 25 µl reaction using two universal bacterial primers for 16s rDNA (Weisburg et al. 1991) (**Table 3.4**). Each 25 µL PCR reaction mix was subjected to thermal cycling consisting of an initial denaturation of 95°C (2 min), followed by 35 cycles of: denaturation at 94°C (30 s), annealing at 55°C (30 s), and extension 72°C (1 min), followed by 9 additional cycles of: denaturation at 94°C (30 s), annealing at 47°C (30 s) and extension at 72°C (1 min, 30 s), with a final extension at 72°C (5 min). The PCR products were electrophoresed in a 1.4% (w/v) agarose gel with TBE buffer at 100 V for 40 min. The gel was stained and examined as before.

3.2.3.5 RAPD (randomly amplified polymorphic DNA) primers

For RAPD, 60 RAPD primers (3 sets, each of 20 10-mer primers: OPA, OPB, OPM - Operon Technologies) (**Table 3.5**) were used to amplify the DNA (**Table 3.3**). Each 25 µL reaction comprised 12.5 µL of GoTaq Green Master mix (Promega), 1 µL (0.6 µM) of each primer, 2 µL of approximately 16 ng DNA template for each sample and 9.5 µL nuclease-free water. A negative control reaction substituted 2 µL nuclease-free water for the DNA. For all RAPD reactions, a G-STORM GT-11426 thermocycler was programmed based on the Manzanares-Dauleux et al. (2001) protocol for initial denaturation at 94ºC for 30 s; followed by 35 cycles of: 94ºC for 30 s, 35ºC for 1 min and 72ºC for 2 min and 30 s; followed by final extension at 72 ºC for 5 min and a holding temperature of 4ºC. The RAPD-PCR products were separated on 1.8% agarose gel and visualised as described earlier. Initially all primers were screened by amplifying a sample from each region. The RAPD primers that amplified at least two bands were then used for all populations. PCR amplification was performed three times to check reproducibility.

Table 3.5: Sixty RAPD primers (3 sets: OPA, OPB, OPM (Operon Technologies), each 10 nucleotides in length, used for RAPD analysis.

Primer number	**Operon Technologies RAPD primer set**		
	OPA	**OPB**	OPM
	Sequence 5´ to 3´	**Sequence 5´ to 3´**	**Sequence 5´ to 3´**
1	CAGGCCCTTC	GTTTCGCTCC	GTTGGTGGCT
2	TGCCGAGCTG	TGATCCCTGG	ACAACGCCTC
3	AGTCAGCCAC	CATCCCCCTG	GGGGGATGAG
4	AATCGGGCTG	GGACTGGAGT	GGCGGTTGTC
5	AGGGGTCTTG	TGCGCCCTTC	GGGAACGTGT
6	GGTCCCTGAC	TGCTCTGCCC	CTGGGCAACT
7	GAAACGGGTG	GGTGACGCAG	CCGTGACTCA
8	GTGACGTAGG	GTCCACACGG	TCTGTTCCCC
9	GGGTAACGCC	TGGGGGACTC	GTCTTGCGGA
10	GTGATCGCAG	CTGCTGGGAC	TCTGGCGCAC
11	CAATCGCCGT	GTAGACCCGT	GTCCACTGTG
12	TCGGCGATAG	CCTTGACGCA	GGGACGTTGG
13	CAGCACCCAC	TTCCCCCGCT	GGTGGTCAAG
14	TCTGTGCTGG	TCCGCTCTGG	AGGGTCGTTC
15	TTCCGAACCC	GGAGGGTGTT	GACCTACCAC
16	AGCCAGCGAA	TTTGCCCGGA	GTAACCAGCC
17	GACCGCTTGT	AGGGAACGAG	TCAGTCCGGG
18	AGGTGACCGT	CCACAGGAGT	CACCATCCGT
19	CAAACGTCGG	ACCCCCGAAG	CCTTCAGGCA
20	GTTGCGATCC	GGACCCTTAC	AGGTCTTGGG

3.2.3.6 Microsatellite primers (ISSR – inter-simple sequence repeats)

For microsatellite primers HKB17/9, HKB 17/33, HKB23/52 and $(GACA)_4$ (**Table 3.6**), a G-STORM (GT-11426) thermocycler was programmed based on protocols from Buhariwalla et al. (1995a) and Faggian (2002). The annealing temperature used was dependent on the primer nucleotide composition and is listed in **Table 3.6.** PCR tube composition was in a total volume of 25 µL as in Buhariwalla et al. (1995a). The cycling was for initial denaturation at 94ºC for 10 min, followed by 40 cycles of: 94ºC for 1 min, the appropriate annealing temperature for 1 min and 72ºC for 2 min; with a final elongation at 72ºC for 5 min and a holding temperature of 4°C (Breslauer et al. 1986; Buhariwalla et al. 1995a; Faggian 2002).

For $(GTG)_5$, PCR composition was as above in a total volume of 25 µL and each included a negative control reaction with 2 µL nuclease-free water instead of the DNA. A G-STORM (GT-11426) thermocycler was programmed based on Ryberg et al. (2011) with a few modifications as listed below. PCR conditions were as follows: an initial denaturation step at

95°C for 15 min; 30 cycles of: denaturation at 92° C for 30 s, annealing temperature 50°C for 30 s, extension at 72° C for 2 min; followed by a final extension step at 72°C for 10 min with a holding temperature of 4°C. The microsatellite PCR products were separated on 2% agarose gel and visualised and analysed as for RAPD primers. All microsatellite PCR reactions were performed three times.

Table: 3.6: Five microsatellite primers used for ISSR analysis.

Primer	**Sequence 5´ to 3´**	**Annealing temperature (°C)**	**References**
HKB 17/9	CGTGGTTCCAATG	36	Buhariwalla et al. (1995); Faggian (2002)
HKB 17/33	GCATCGTCTG	36	Buhariwalla et al. (1995a); Faggian, (2002)
HKB 23/52	CGTGCGTACTTAGG	39	Buhariwalla et al. (1995a); Faggian, (2002)
$(GACA)_4$	GACAGACAGACAGACA	38	Buhariwalla et al. (1995a); Faggian (2002)
$(GTG)_5$	GTGGTGGTGGTGGTG	50	Ryberg et al. (2011)

3.2.4 Cluster analysis

Polymorphism of consistent bands was scored manually based on presence (1) or absence (0) of bands of different sizes for each primer and entered manually into an Excel spreadsheet as a similarity matrix along with results from plant hosts and fungi. This was used to perform Principal Components analysis and to generate a dendrogram using hierarchical cluster analysis, both at p=0.05, using the statistical program Minitab Version 16 (Minitab Inc.).

3.3 Results

3.3.1 Determination of purity and quantity of genomic DNA

DNA extracted from all galls of the different populations gave large and acceptable quantities of DNA (**Fig. 3.3**), as did the common host plants and contaminant fungi.

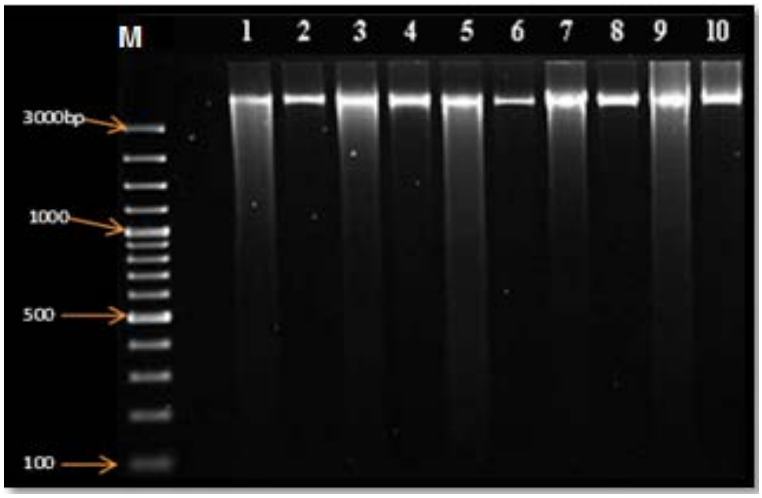

Figure 3.3: Genomic DNA of ten populations of *Plasmodiophora brassicae*. Lane M molecular weight marker (GeneRuler), then (left to right): Population 1 (Boisdale – VIC), Population 2 Woori Yallock – VIC, Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

3.3.2 Universal primers ITS-1 and ITS-4

Genomic DNA of the ten *P. brassicae* populations amplified using universal primers ITS-1 and ITS-4 gave a PCR product of ~700 bp (red oval) (**Fig. 3.4**). The PCR was repeated for these samples and always showed the same result.

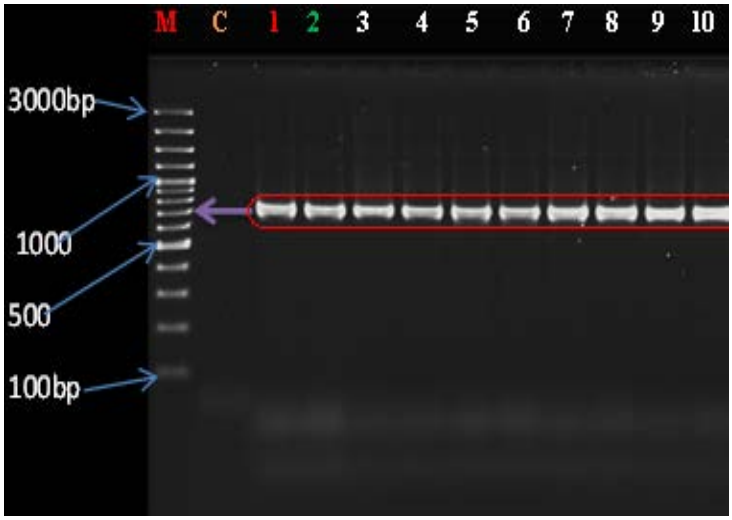

Figure **3.4**: PCR products from amplification of *Plasmodiophora brassicae* populations using ITS-1 and ITS-4 primers. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

3.3.3 Specific primers (PbITS1 and PbITS2) and (PbITS6 and PbITS7)

The amplifications of *P. brassicae* DNA from galls of infected plants with specific primers PbITS1 and PbITS2 resulted in single products of the predicted size of ~1100 pb (yellow oval) (**Fig. 3.5**). Subsequently the nested primers PbITS6 and PbITS7 resulted in clear single bands of the predicted size of ~620 bp (yellow oval) (**Fig. 3.6**) Field-collected samples of soil/water (Chapter 2) also resulted in clear single bands of ~620 bp.

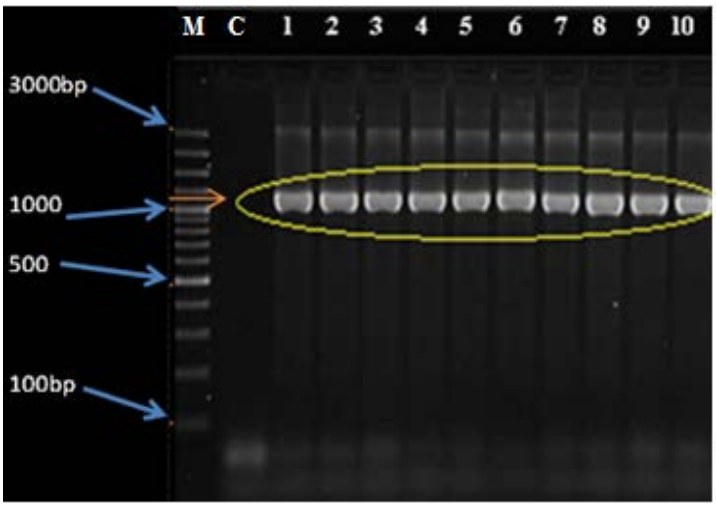

Figure 3.5: PCR products from amplification of *Plasmodiophora brassicae* populations using specific primers PbITS1 and PbITS2. Lanes are numbered from left to right. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow-Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

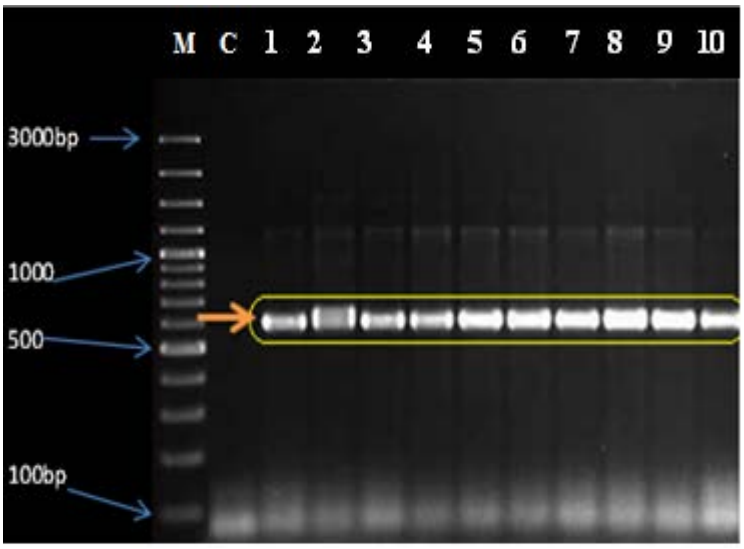

Figure 3.6: PCR products from amplification of *Plasmodiophora brassicae* populations using PbITS6 and PbITS7. Lanes are numbered from left to right. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

3.3.4 Fungi-specific primer ITS-1F with universal primer ITS-4

DNA from both fungi (*Exophiala dermatitidis* and *Penicillium chrysogenum*) produced bands of the expected size (~550 bp and ~600 bp respectively) (yellow oval) with primers ITS-1F and ITS-4. None of the gall DNA extracts produced a band (**Fig. 3.7**)

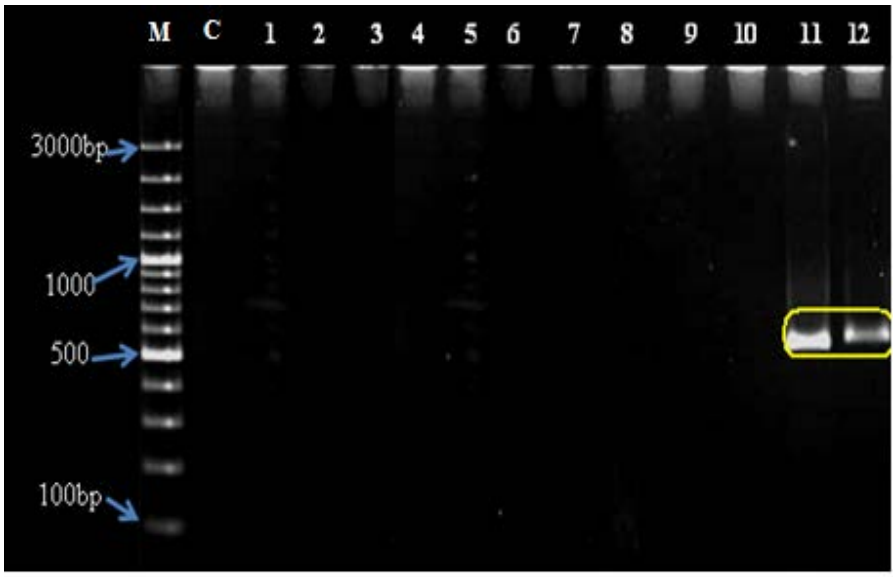

Fig. 3.7: PCR products from amplification of fungi and galls using ITS-1F and ITS-4. Lanes are numbered left to right: Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA, 11 DNA of *Exophiala dermatitidis*, and 12 DNA of *Penicillium chrysogenum*. Note the presence of bands of ~550 bp and ~600 bp respectively from *Exophiala dermatitidis* and *Penicillium chrysogenum* respectively (yellow oval).

3.3.5 Bacteria-specific primers fD1 and rP2

DNA from the bacterium *E. coli* produced a band of the expected size ~1400bp with fD1 and rP2 primers (yellow oval). None of the gall DNA extracts produced a band (**Fig. 3.8**)

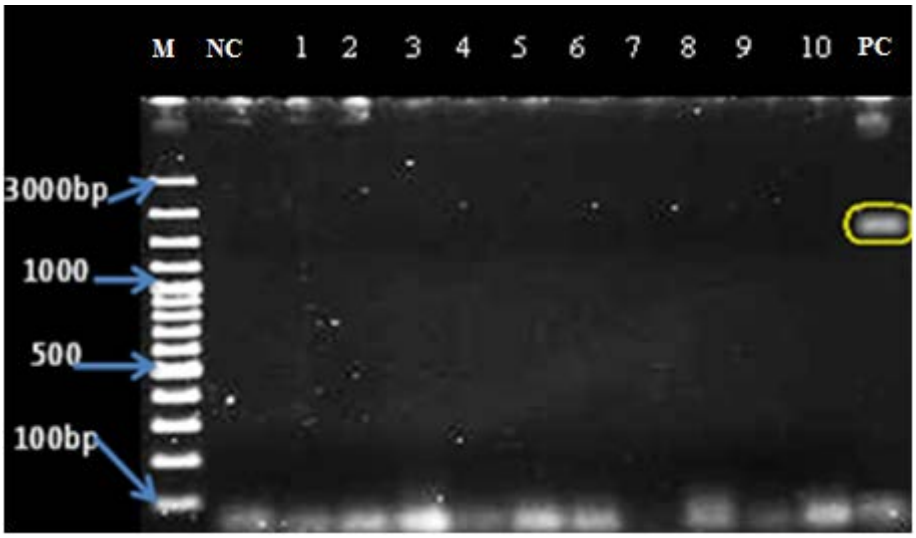

Fig. 3.8: PCR products from amplification of bacteria and galls using primers fD1 and rP2. Lanes are numbered left to right: Lane M molecular weight marker (GeneRuler), lane NC negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA, and PC (positive control) DNA of Bacteria (*Escherichia coli).* Note the presence of a ~1400 bp band corresponding to *Escherichia coli* (yellow oval).

3.3.6 RAPD primers

All ten populations of *P. brassicae* gave amplification products with banding patterns after RAPD amplification with 20 each of the OPA, OPB and OPM primers (**Tables 3.5**). OPA primers produced 369 scorable bands for the ten different populations, while OPB primers generated 383 bands and OPM primers generated 414 bands (**Table 3.7**). A summary of the reactions is presented in **Table 3.8**. In general, the RAPD reactions produced sufficient information for a study of the genetic diversity of the populations of *P. brassicae*.

Table 3.7: Polymorphism from RAPD analysis of populations of *Plasmodiophora brassicae*.

Operon primer	Total bands for all populations	Range of band sizes (bp)	Primer with most polymorphism
OPA set	369	180-3200	OPA-17
OPB set	383	200-3400	OPB-11
OPM set	414	200-3500	OPM-07

Table 3.8: Summary of polymorphism detected using RAPD primers from Operon kits OPA, OPB and OPM amongst ten populations of *Plasmodiophora brassicae*, where: (+++) much polymorphism, (++) moderate polymorphism, (+) little polymorphism, (-) no polymorphism, N/A: no clear reaction, N/A* no reaction.

Primer number	Polymorphism		
	OPA	OPB	OPM
1	++	+	+
2	+	++	+
3	+	+++	+
4	-	++	N/A*
5	-	-	-
6	N/A*	+	N/A*
7	+	+	+++
8	++	+	+
9	N/A*	N/A	+
10	+	N/A	-
11	++	++	+
12	++	+	+
13	+	+	+
14	N/A	+	N/A
15	N/A	N/A	N/A
16	+	N/A	+
17	++	-	N/A
18	+	+	++
19	-	-	+

3.3.6.1 OPA primers

Only 13 OPA primers produced reproducible clear bands and showed polymorphism for all populations, i.e. OPA-01, OPA-02, OPA-03, OPA-07 OPA-08, OPA-10, OPA-11, OPA-12, OPA-13, OPA-16, OPA-17, OPA-18 and OPA-20 (**Figs 3.9 – 3.13**). The greatest amount of polymorphism was with OPA-17 (**Fig. 3.13a**).

All populations produced strong consistent polymorphic bands (red ovals). In particular, population 3 was markedly different from the other populations with OPA-01, OPA-02, OPA-08, OPA-10, OPA-11, OPA-12, OPA-16, OPA-17 and OPA -20. Population 6 was different from the others with OPA-1, OPA-8, OPA-11 and OPA-12. Population 10 was different from the others with OPA-01, OPA-08, OPA-12, and OPA-20.

Populations were distinguished by unique bands. For example, populations 2 and 5 had a unique band of ~900 bp with OPA-01 (**Fig. 3.9a**) (yellow squares). Similarly; population 3 had a unique band of ~500 bp with OPA-15 (**Fig. 3.12c**) and double bands at ~480 bp and ~540 bp respectively with OPA-17 (**Fig. 3.13a**) (yellow square). Populations 6 and 8 shared a band of ~700 bp with OPA-08 (**Fig. 3-10c**) (yellow square).

Some primers produced unique bands for one or a few populations. For instance, OPA-01 amplified a strong unique band at ~700 bp for only populations 3 and 10 (**Fig. 3.9a**) (green circle), whereas no such band was observed for the other populations. Moreover, OPA-08 amplified double clear unique bands of~ 300 bp and ~400 bp only for populations 3, 9 and 10 (**Fig. 3.10c**) (green circles).

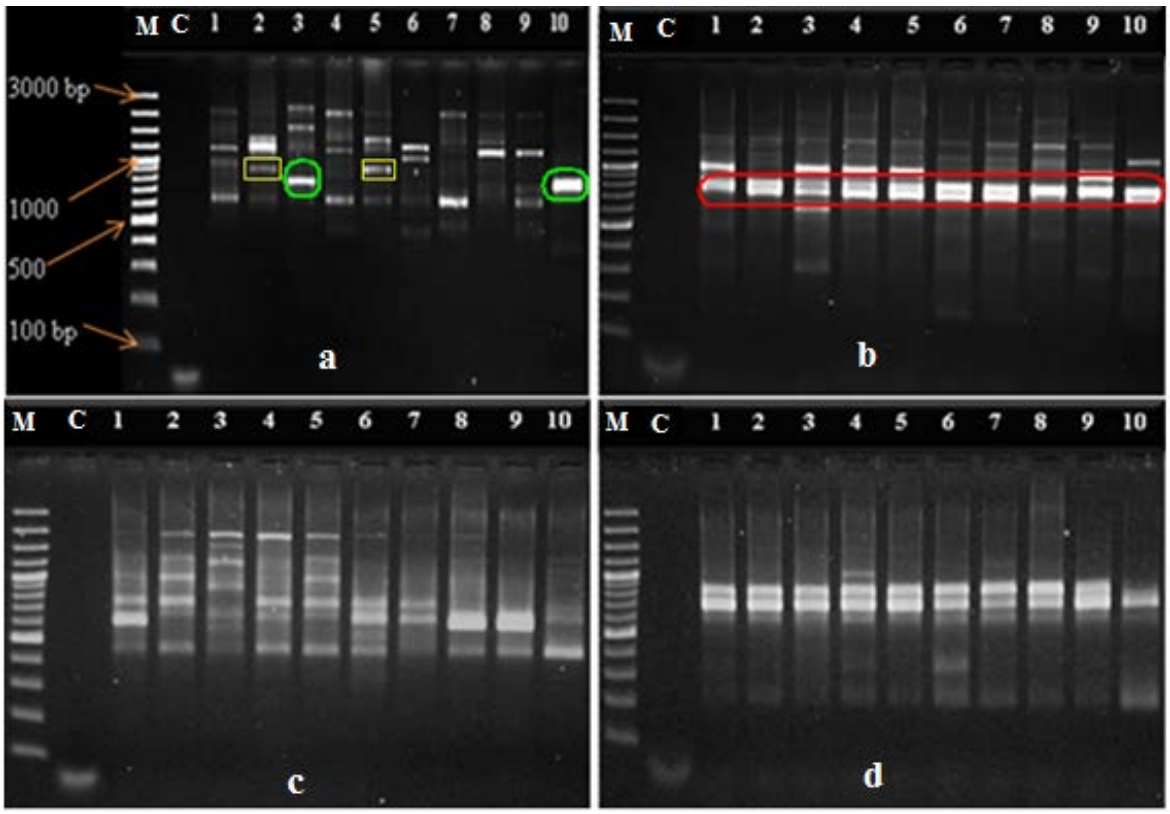

Figure: 3.9: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPA primers. (a) OPA-01, (b) OPA-02, (c) OPA-03 and (d) OPA-04. genomic Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

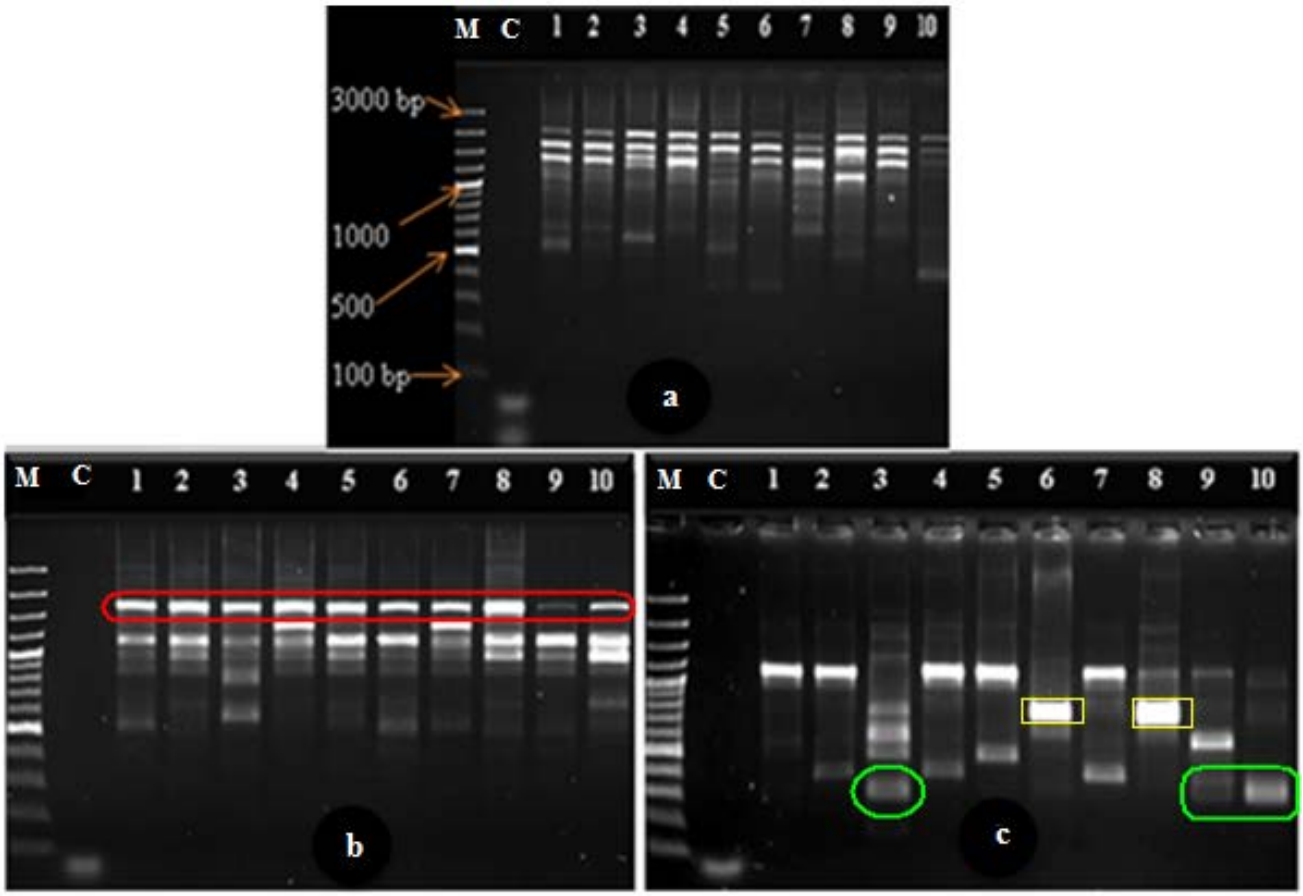

Figure 3.10: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPA primers. (a) OPA-05, (b) OPA-07 and (c) OPA-08. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

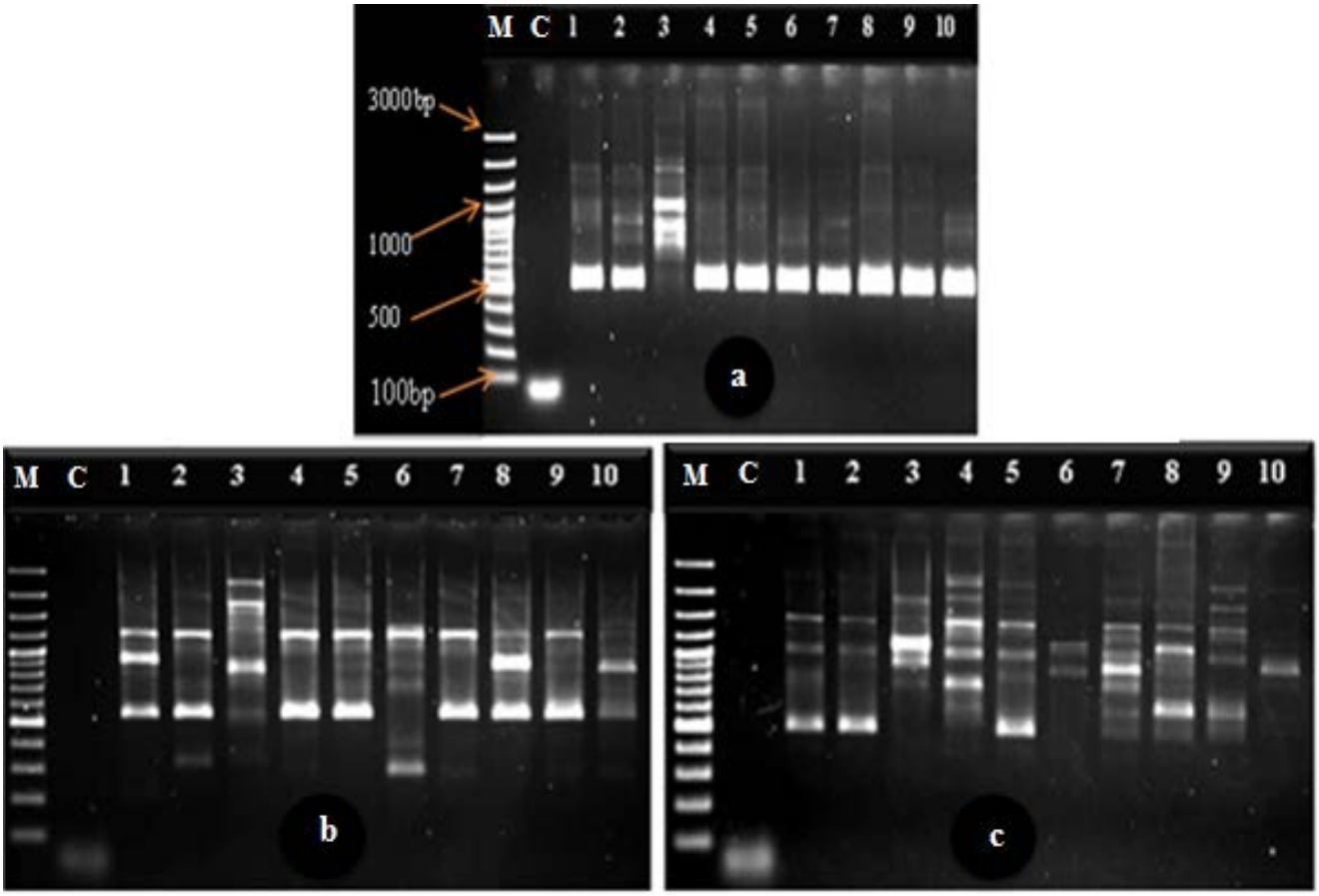

Figure 3.11: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPA primers. (a) OPA-10, (b) OPA-11 and (c) OPA-12. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

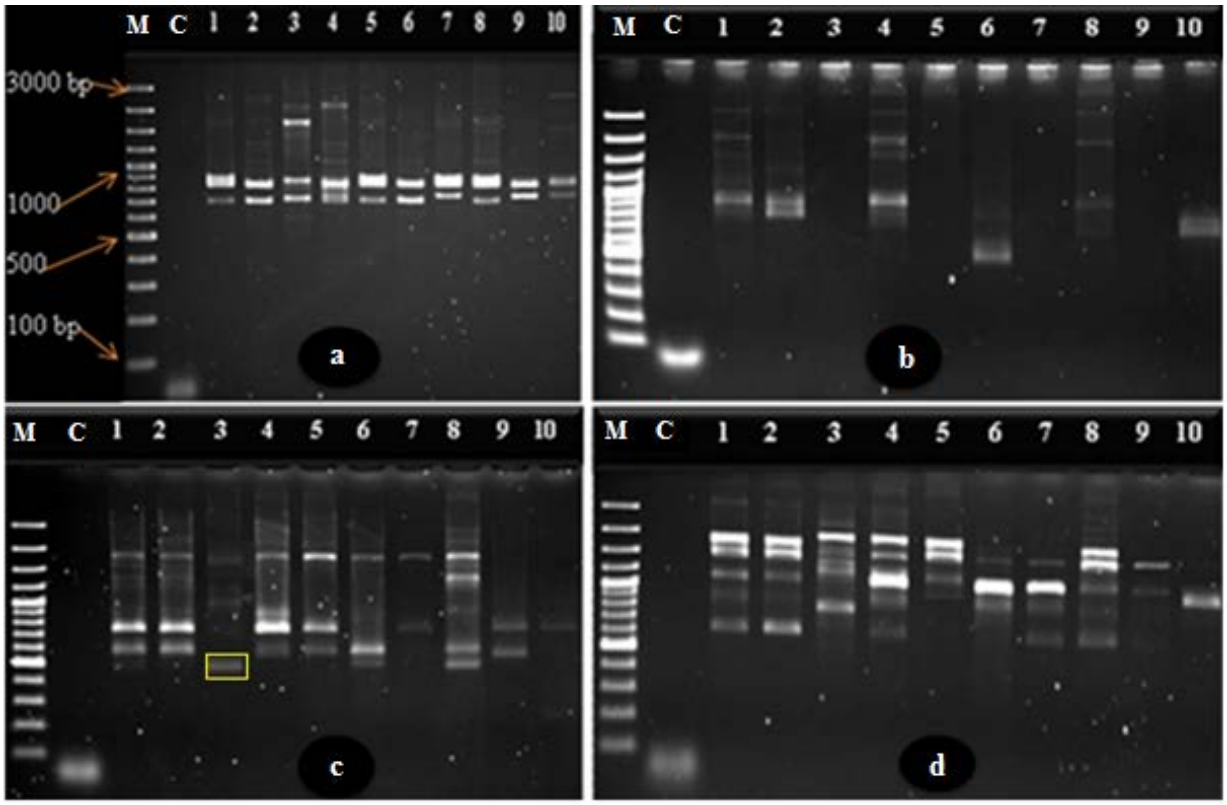

Figure 3.12: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPA primers. (a) OPA-13, (b) OPA-14, (c) OPA-15 and (d) OPA-16. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

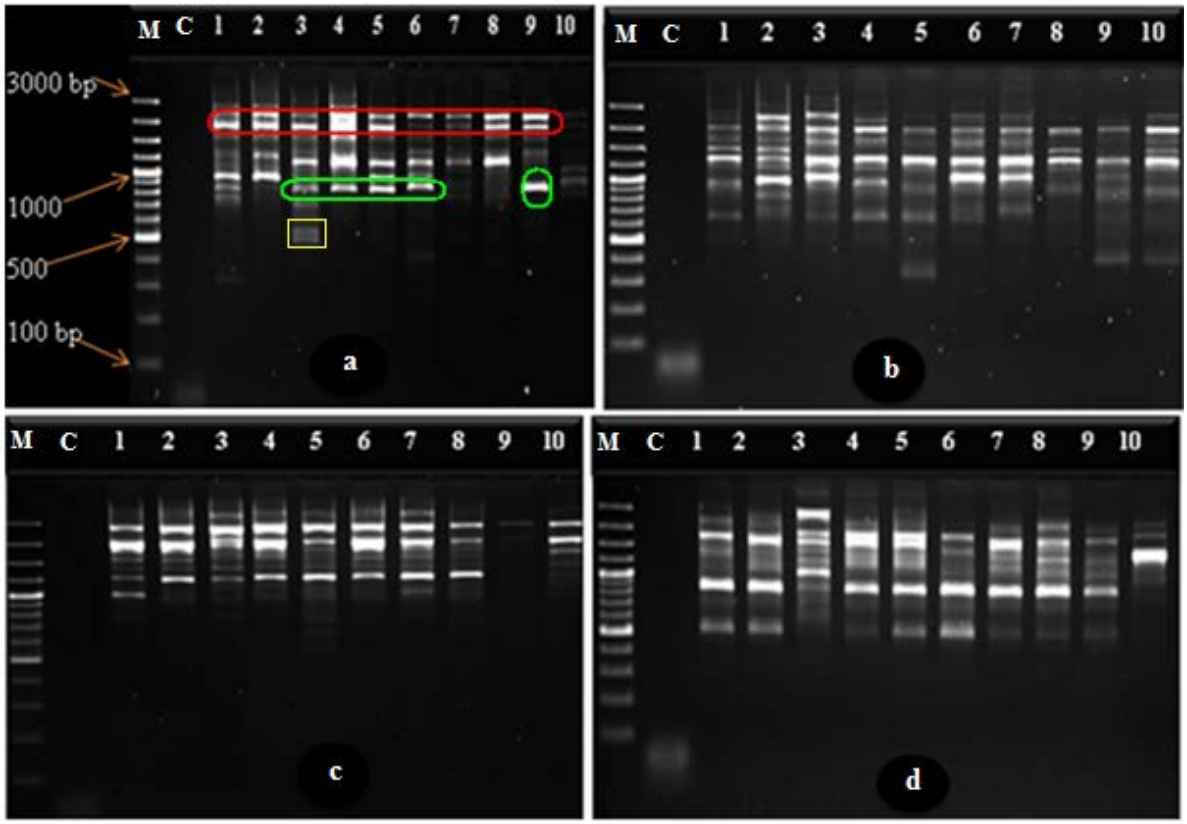

Figure 3.13: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPA primers. (a) OPA-17, (b) OPA-18, (c) OPA-19 and (d) OPA-20. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

3.3.6.2 OPB primers

All OPB primers produced strong reproducible bands. Only 12 OPB primers reacted with all test populations, generating reproducible clear bands showing polymorphism, i.e. OPB-01, OPB-02, OPB-03, OPB-04 OPB-06, OPB-07 OPB-08, OPB-11, OPB-12, OPB-13, OPB-14 and OPB-18 (**Figs 3.14-3.18**).

There was clear polymorphism in the populations; populations 1, 2, 3, 6 and 10 had consistently different profiles from the other populations 4, 5, 7, 8 and 9. In particular, population 1 showed a unique polymorphic band of ~3000 bp (yellow box) with OPB-18 (**Fig. 3.18b**). Population 4 showed a unique thick band of ~600 bp with OPB-03 (**Fig. 3.14 c**). The appearance of Population 3 was very different from those of the other populations with OPB-02, OPB-03, OPB-4, OPB-06, OPB-10, OPB-11, OPB-12, OPB-13, and OPB-14. Population 7 had a unique band of ~ 420 bp with OPB-03 (**Fig. 3.14c**). Population 10 was unique, with a band of ~240 bp with OPB-07 (**Fig. 3.15c**), a band of ~850 bp with OPB-11 (yellow color) (**Fig. 3.16 c**) and a band of ~700 bp with OPB-18 (yellow box) (**Fig. 3.18b**).

The primers OPB-03 (**Fig. 3.14c**), OPB 11 (**Fig. 3.16c**) and OPB-18 (**Fig. 3.18b**) produced clear polymorphisms with all ten populations (red box), while some primers produced unique bands for some individual populations, e.g. population 2 with OPB-02; population 3 with OPB-02, OPB-03, OPB-04, OPB-10, OPB-11, OPB-12, OPB-13, OPB-14, OPB-15 and OPB-20; population 10 with OPB-01, OPB-03, OPB-05, OPB-07, OPB-11, and OPB-14 OPB-17 and OPB-18.

Particular primers produced unique bands for a few populations; for example, OPB-01 amplified a strong unique band at ~340 bp for population 9 (**Fig. 3.14a**). For population 3, OPB-12 amplified a strong unique band at ~420 bp (**Fig. 3.16d**) and all but population 3 had strong single bands with OPB-10 (~800 bp) (green ovals) (**Fig. 3.16b**), OPB-11 (~1100 bp) (**Fig. 3.16c**) and OPB-13 (~1800 bp) (**Fig. 3.17a**).

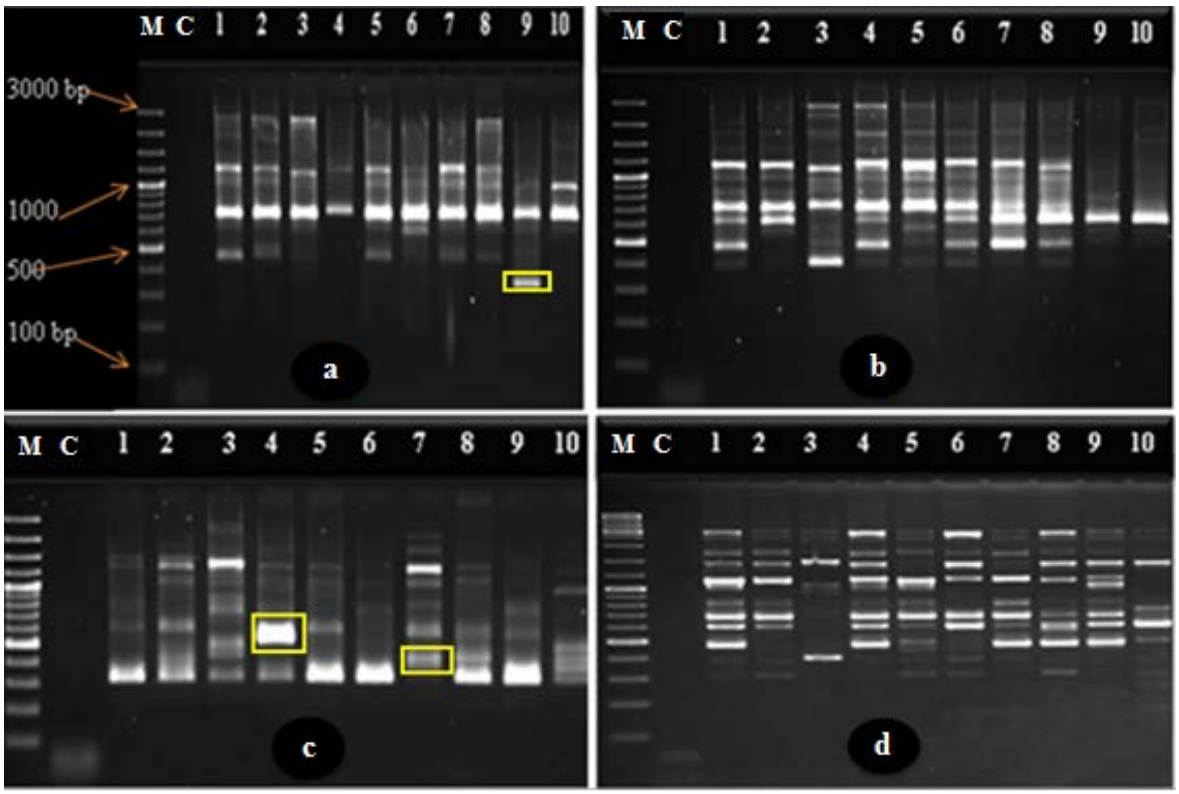

Figure 3.14: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPB primers. (a) OPB-01, (b) OPB-02, (c) OPB-03 and (d) OPB-04. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

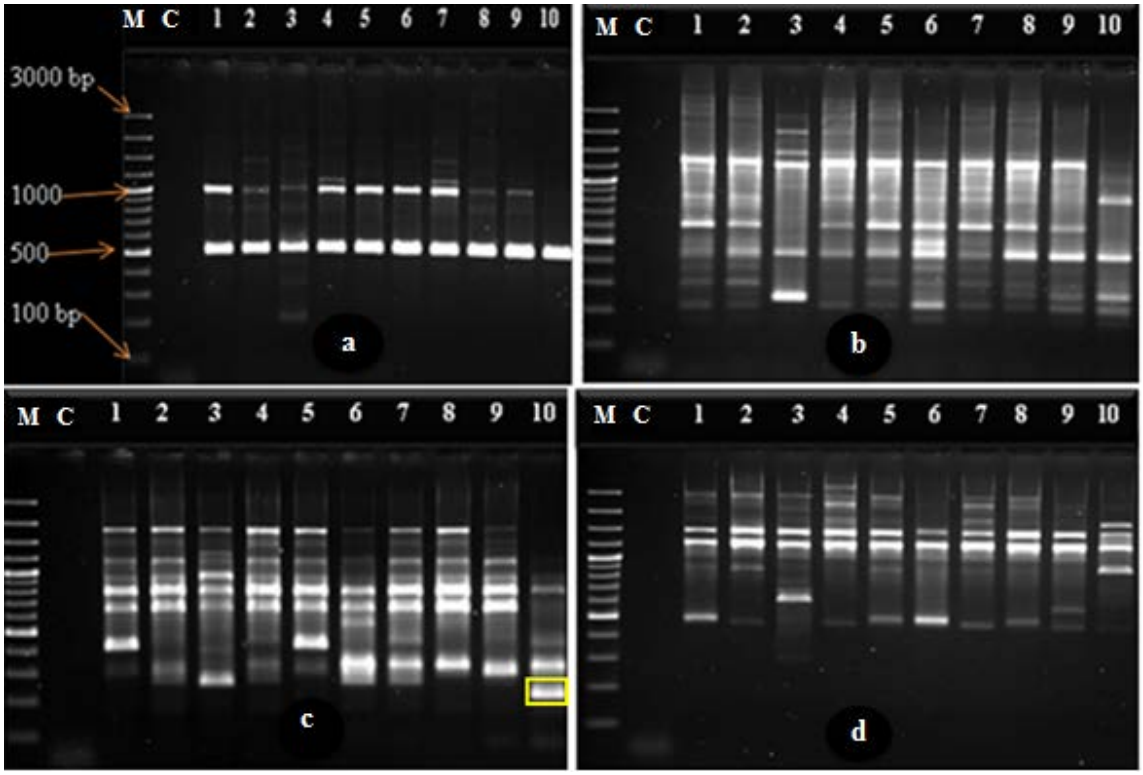

Figure 3.15: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPB primers. (a) OPB-05,(b) OPB-06, (c) OPB-07 and (d) OPB-08. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

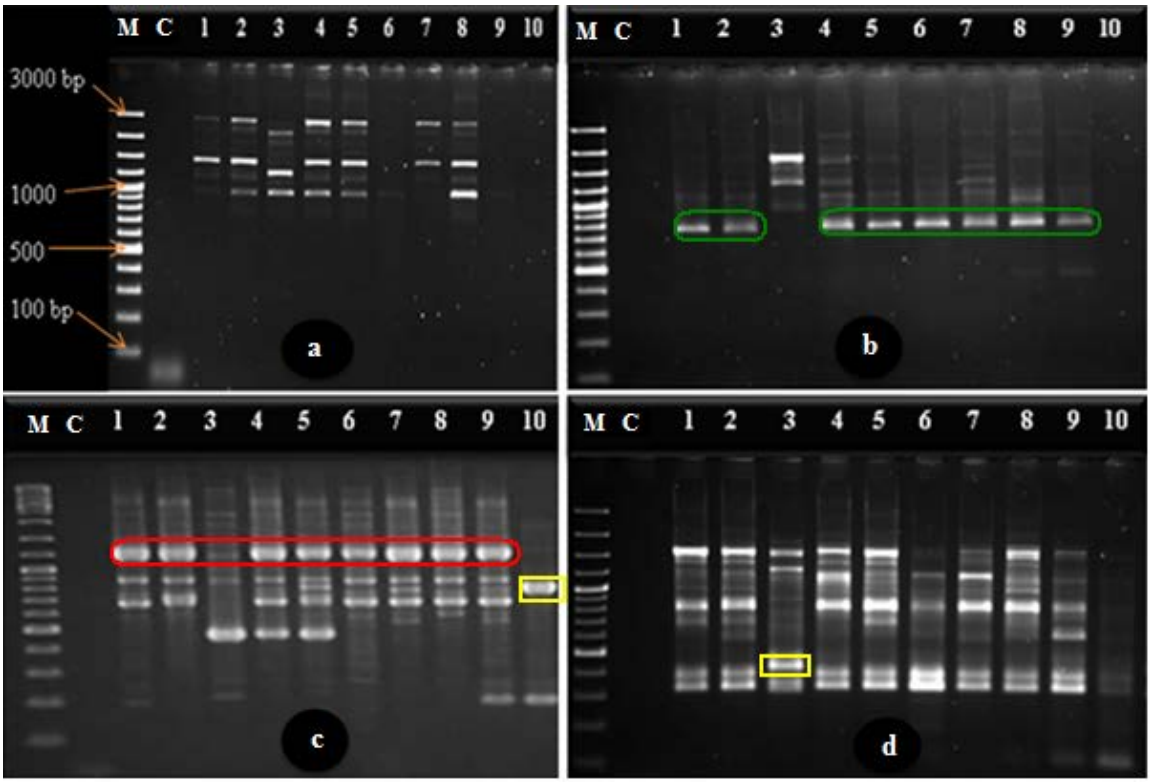

Figure 3.16: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPB primers. (a) OPB-09, (b) OPB-10, (c) OPB-11 and (d) OPB-12. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

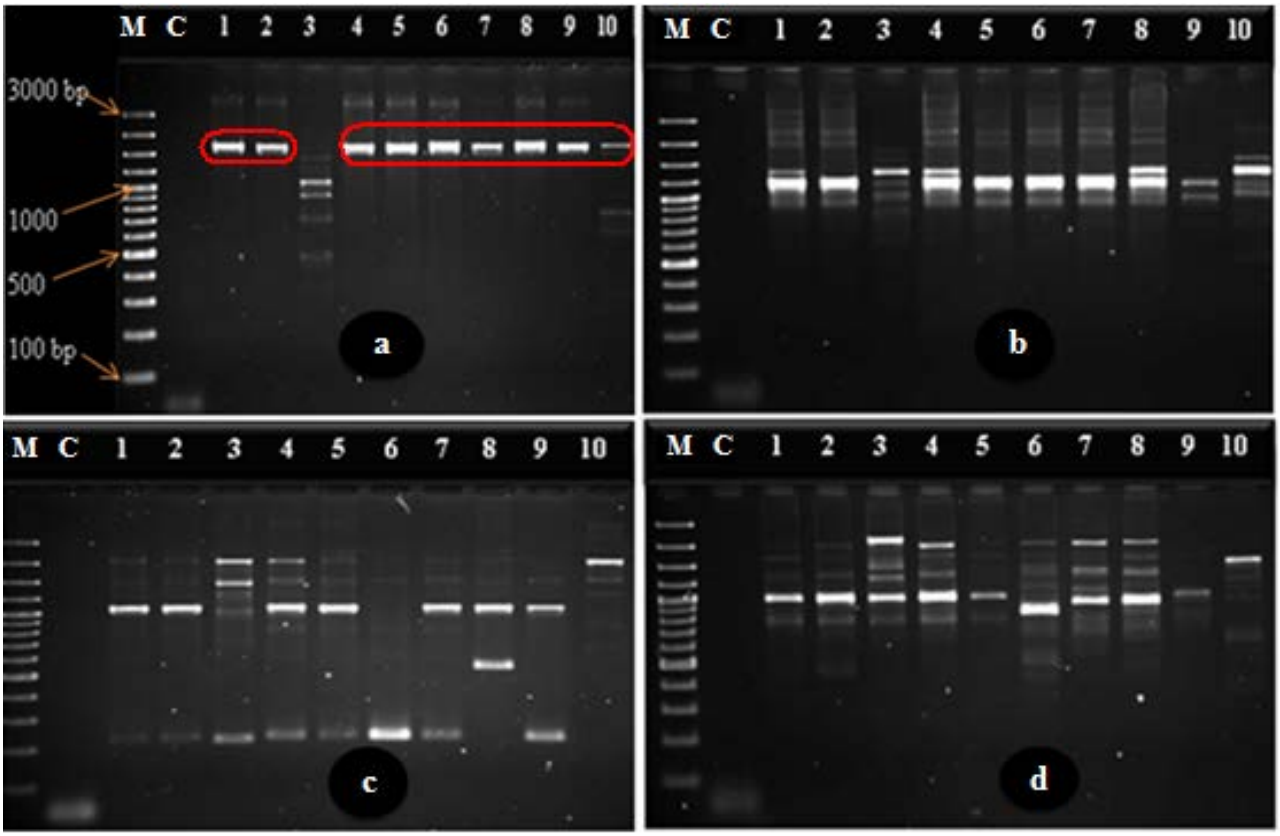

Figure 3.17: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPB primers. (a) OPB-13, (b) OPB-14, (c) OPB-15 and (d) OPB-16. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

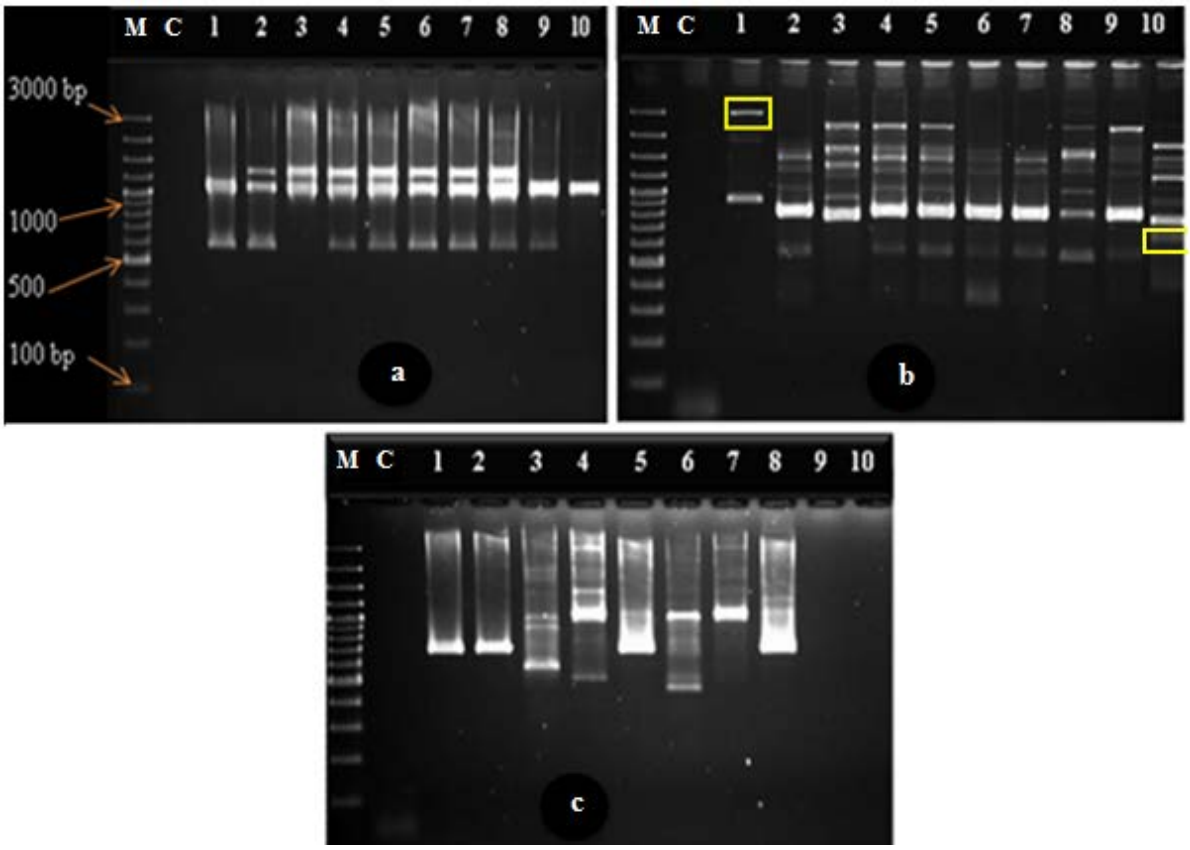

Figure 3.18: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPB primers. (a) OPB-17, (b) OPB-18 and (c) OPB-20. Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

3.3.6.3 OPM primers

Eighteen OPB primers produced different polymorphism bands, with varying degrees of response and interaction. Only 13 OPB primers reacted with all test populations, generating reproducible clear bands showing polymorphism, i.e. OPM-01, OPM-02, OPM-03, OPM-07, OPM-08, OPM-09, OPM-11, OPM-12, OPM-13, OPM-16, OPM-18, OPM-19 and OPM-20 (**Figs 3.19-3.23**).

Thirteen OPM primers generated 2-13 polymorphic bands of ~200-3500 bp. The least polymorphism was observed with primers OPM-08 and OPM-10 and the greatest with primers OPM-01 and OPM-07. The OPM-04 and OPM-06 primers did not produce any product with any sample in each of the three repeats.

All ten *P. brassicae* populations reacted with these fourteen OPM primers and showed clear polymorphic reactions, especially with OPM-O1 (**Fig. 3.19a**), OPM-13 (**Fig. 3.22a**) and OPM-19 (**Fig. 3.23c**). Population 3 showed many differences from other populations, as shown with OPM-01, OPM-02, OPM-04, OPM-06, OPM-12, OPM-13 and OPM-16. Specific unique bands were noted for some populations with some primers; for instance, population 3 had a unique band of ~1300 bp (yellow box) (**Fig. 3.19b**) with OPM-02, while population 10 had a unique band of ~600bp with OPM-08 (yellow box) (**Fig. 3.20c**). Other primers produced more uniform reactions, e.g. a common band of ~700 bp with OPM-11 (red box) (**Fig. 3.21c**).

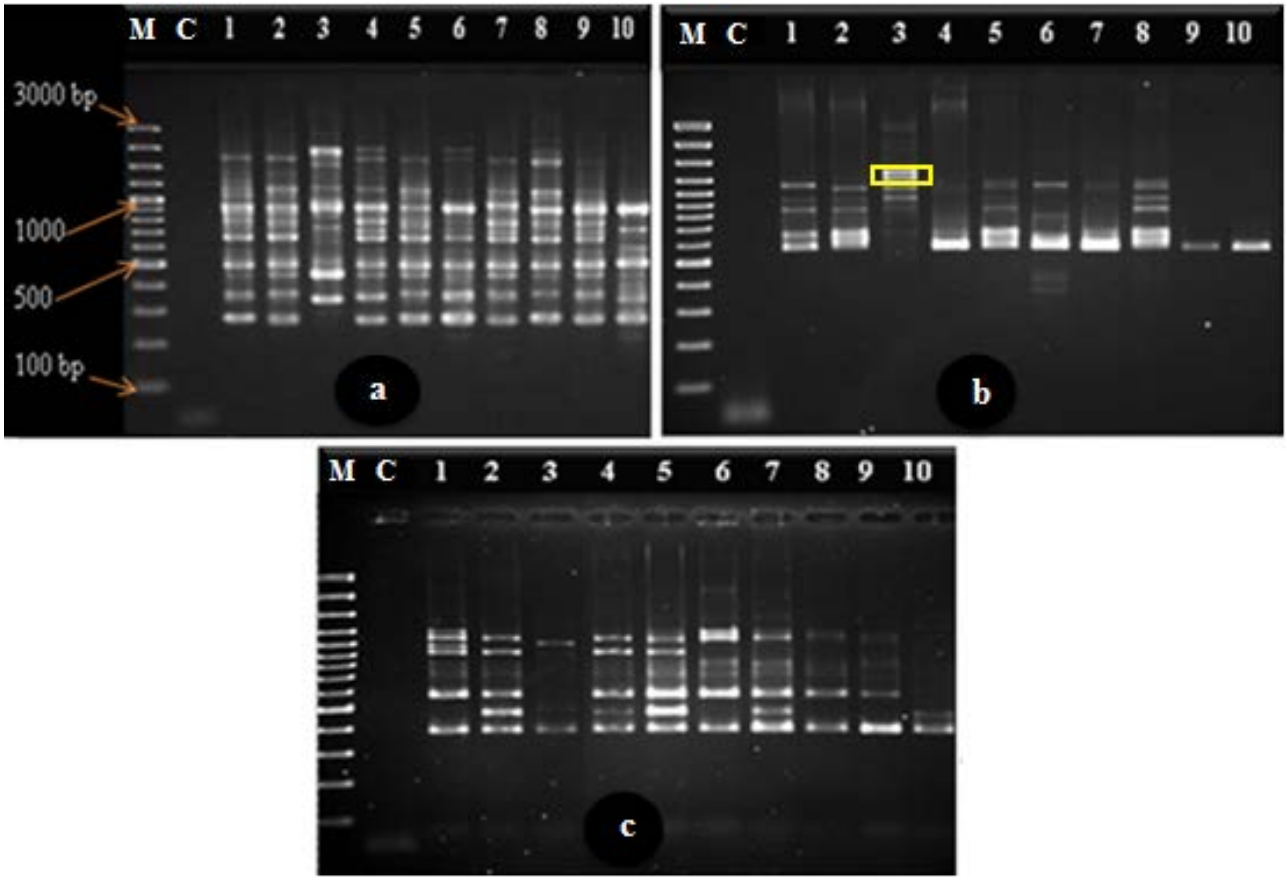

Figure 3.19: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPM primers. (a) OPM-01, (b) OPM-02 and (c) OPM-03. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

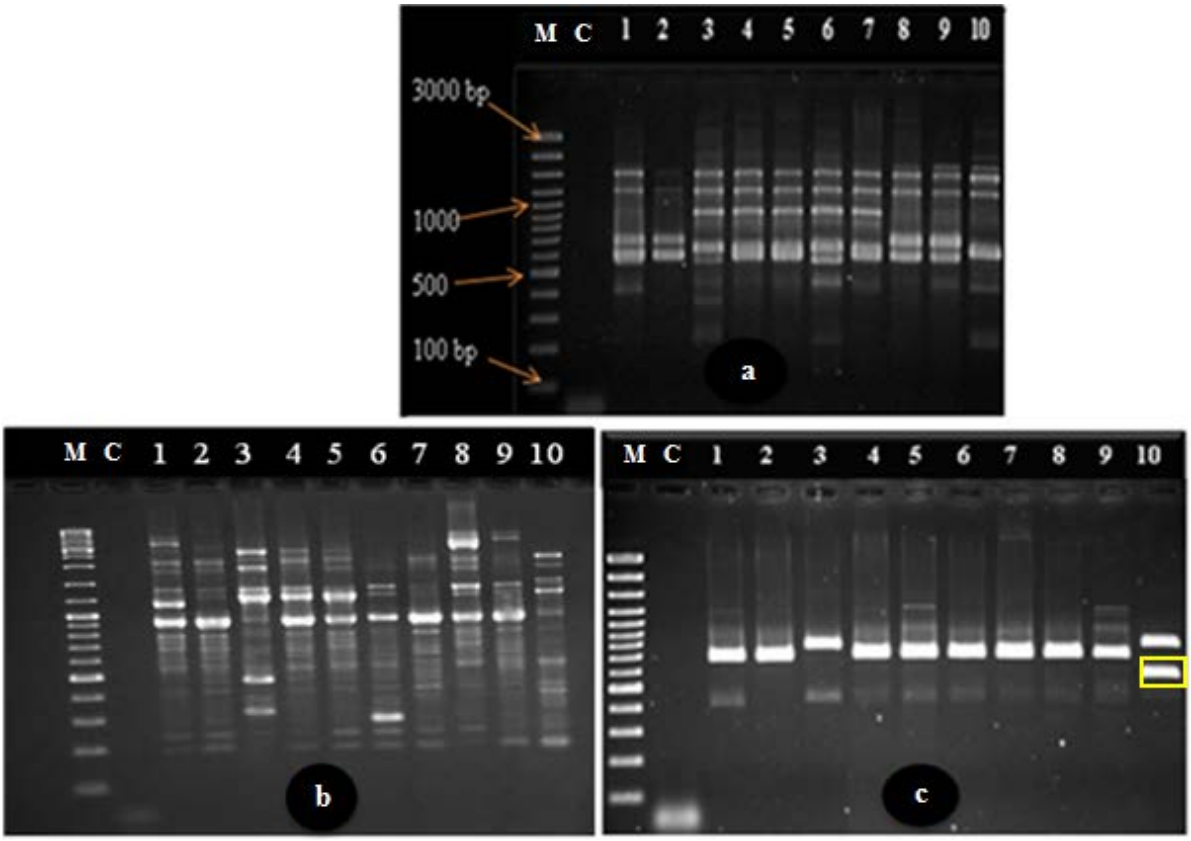

Figure 3.20: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPM primers (a) OPM-05, (b) OPM-07 and (c) OPM-08. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

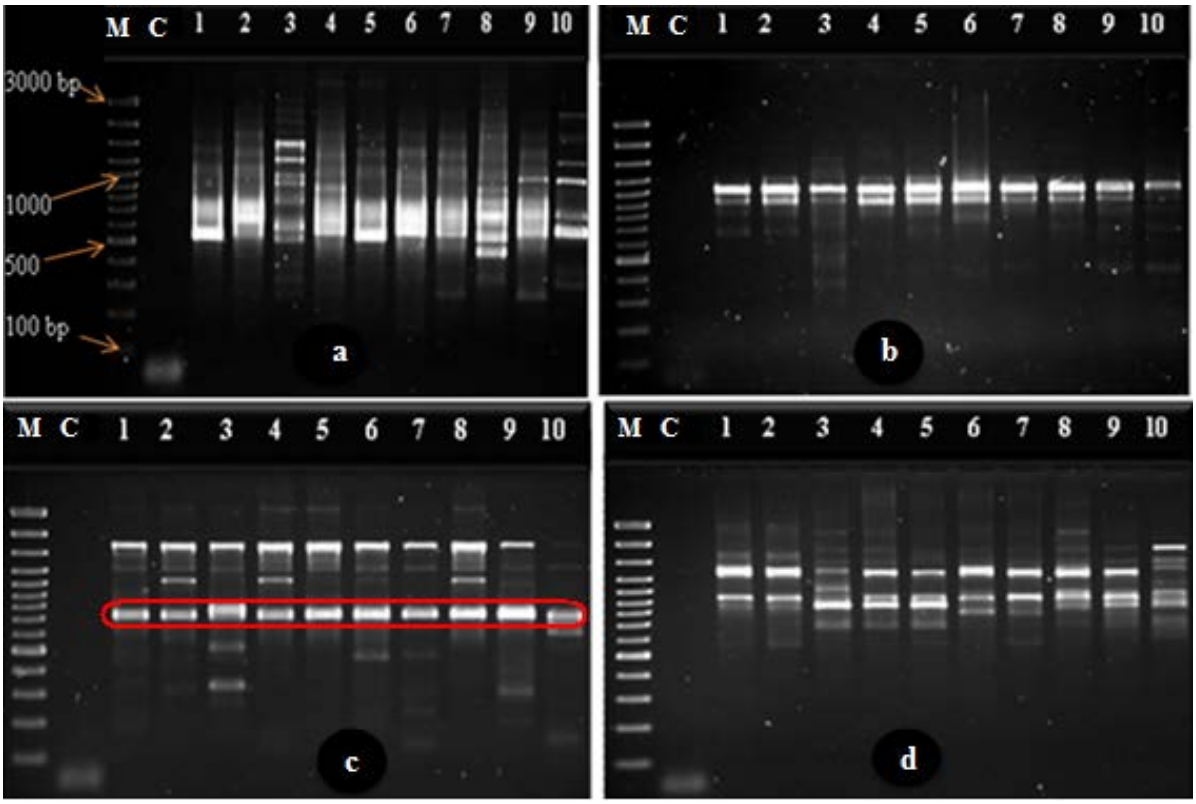

Figure 3.21: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPM primers (a) OPM-09, (b) OPM-10, (c) OPM-11 and (d) OPM-12. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

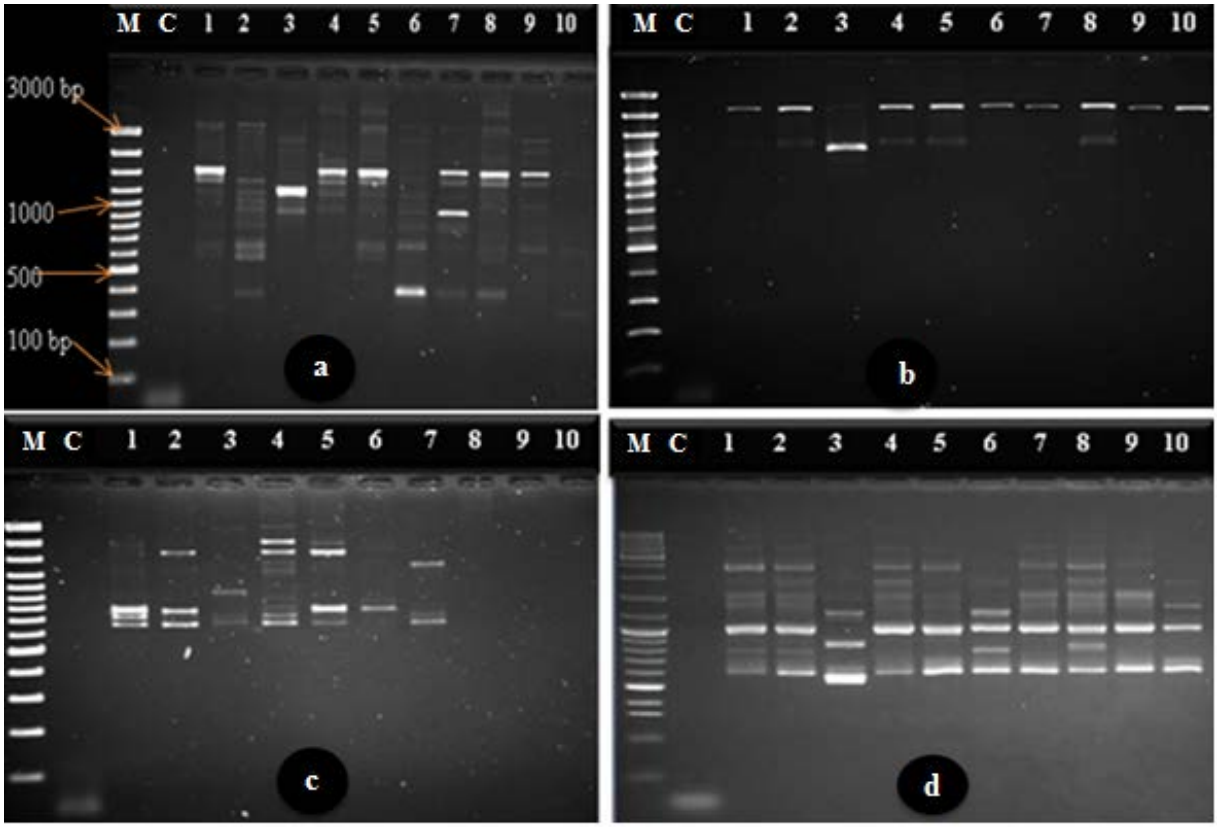

Figure 3.22: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPM primers (a) OPM-13, (b) OPM-14, (c) OPM-15 and (d) OPM-16. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

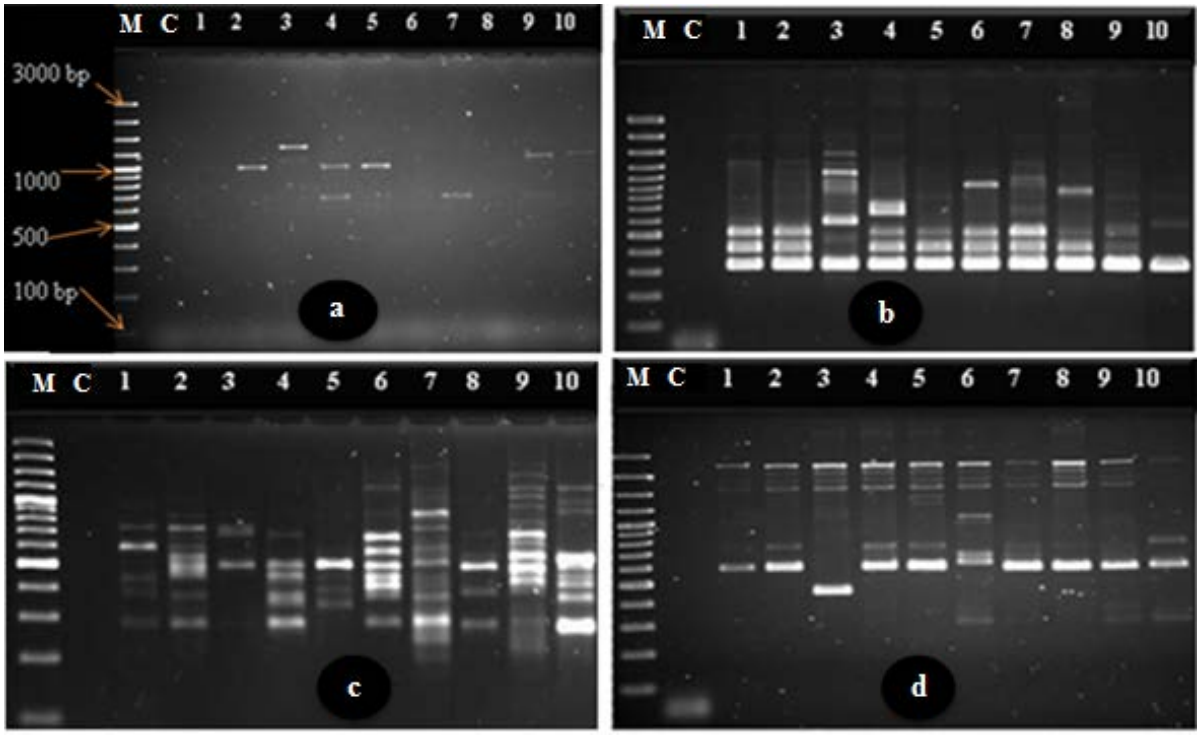

Figure 3.23: RAPD-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with OPM primers (a) OPM-17, (b) OPM-18, (c) OPM-19 and (d) OPM-20. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

3.3.7 Microsatellite primers

All of the populations of *P. brassicae* gave banding patterns of amplification products with all of the microsatellite primers (**Figs. 3.24-3.25)**

The primer HKB 17/9 fingerprints yielded 2-14 bands of ~260- 3200 bp, while HKB 17/33 yielded 5-13 bands of ~180-3000 bp and HKB23/52 yielded 1-16 bands of ~200-3100 bp. $(GACA)_4$ produced 7-13 bands of ~ 280-3200 bp and $(GTG)_5$ yielded up to 11 bands, of ~380-3000 bp.

HKB 17/9 amplified two bands with population 3, at 600 and 700 bp respectively, that were absent from the rest of the populations (**Fig. 3.24a**) (yellow box). A polymorphic band of ~330 bp appeared with HKB 23/52 for populations 2, 4, 5, 7, 9 and 10 (Fig. **3.24c**) (green ovals). $(GACA)_4$ produced a polymorphic fragment of ~300 bp that differentiated population 3 from the others (Fig. **3.25a**) (red box).

The degree of polymorphism is summarized in Table (**3.9**). Three of the five primers - HKB17/33, HKB23/52 and $(GACA)_4$ - were the most useful, as they showed polymorphism for all ten populations.

Table 3.9: Summary of polymorphism detected using microsatellite primers with ten *Plasmodiophora brassicae* populations from Victoria and Western Australia, where: (**+++)** much polymorphism, (++) moderate polymorphism, (+) little polymorphism**.**

Primer name	Polymorphism
HKB17/9	++
HKB17/33	+++
HKB23/52	+++
$(GACA)_4$	+++
$(GTG)_5$	+

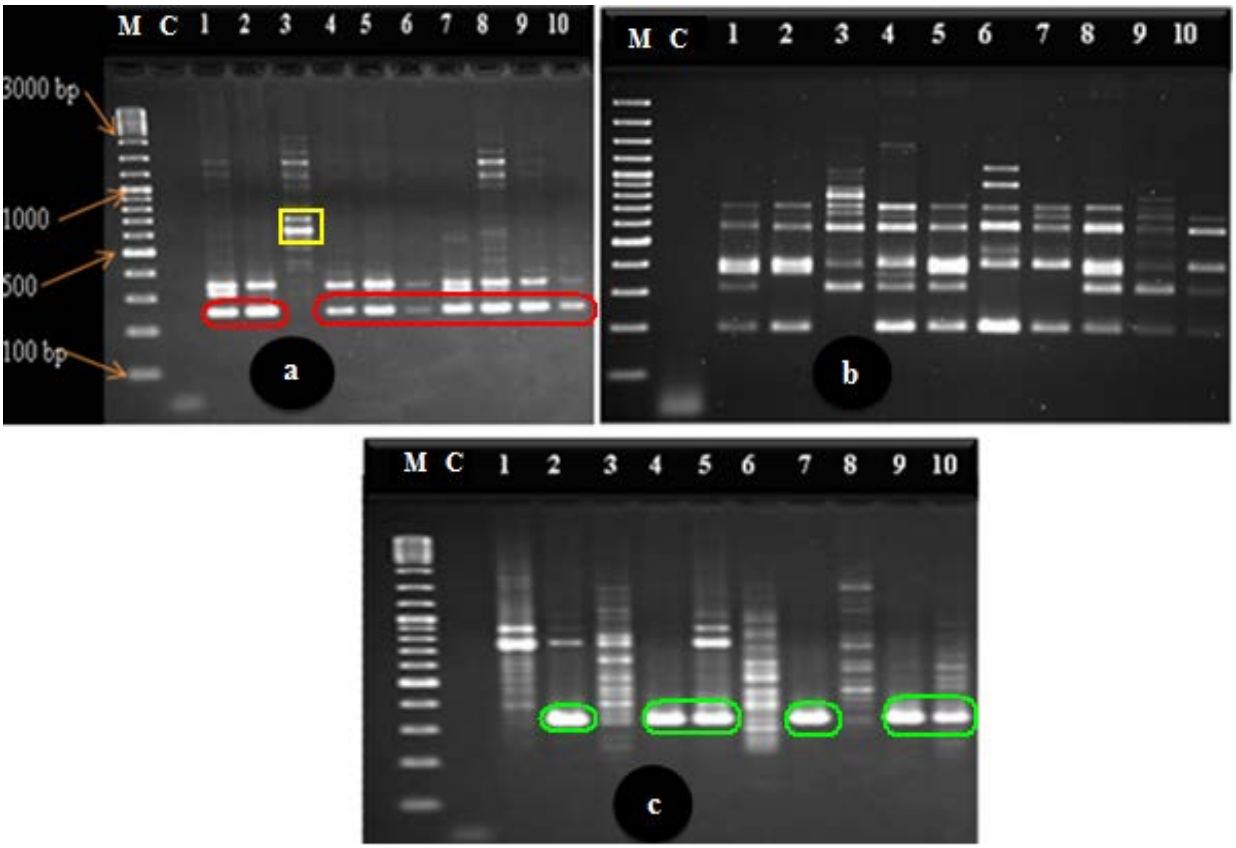

Figure 3.24: Microsatellite-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with microsatellite primers. (a) HKB 17/9, (b) HKB 17/33 and (c) HKB 23/52. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

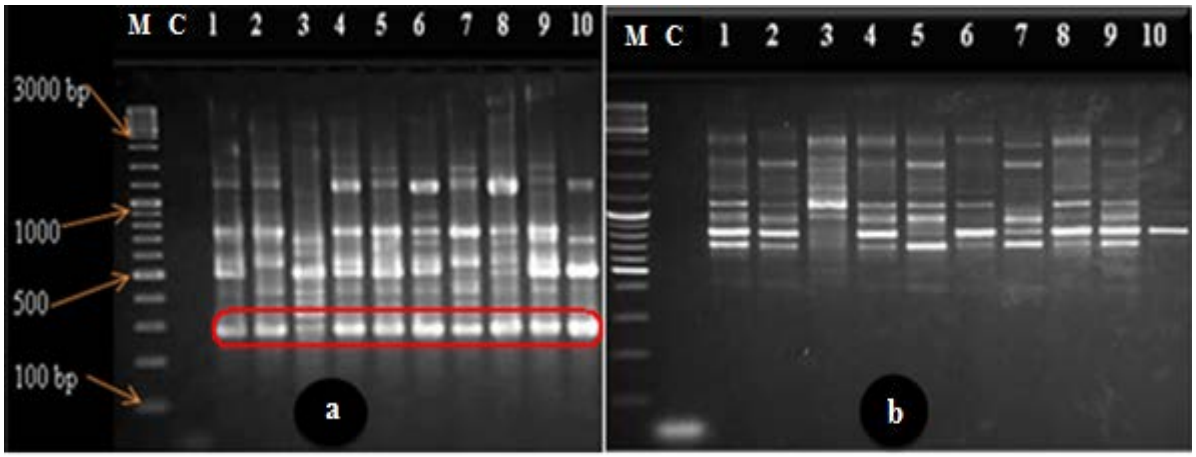

Figure: 3.25: Microsatellite-PCR products from genomic DNA of ten populations of *Plasmodiophora brassicae* amplified with microsatellite primers. (a) $(GACA)_4$ and (b) $(GTG)_5$. Lane M molecular weight marker (GeneRuler), lane C negative control (water). Lanes are (left to right): Population 1 (Boisdale – VIC), Population 2 (Woori Yallock – VIC), Population 3 (Devon Meadows – VIC, Population 4 Rosebud- VIC, Population 5 Trentham- VIC, Population 6 Cora Lynn Bunyip- VIC, Population 7 Werribee - VIC, Population 8 Lindenow- Bairnsdale – VIC, Population 9 Manjimup – WA, Population 10 Manjimup – WA.

3.3.8 Summary and cluster analysis of genetic diversity studies

Five primers were the most useful for detecting polymorphism in the ten populations of *P. brassicae* examined (**Table 3.10**). These were used subsequently to examine genetic stability during successive generations and of mixing populations (Chapter 4).

Table 3.10: Most polymorphic primers from RAPD and microsatellite analysis of populations of *Plasmodiophora brassicae*.

Primer	**Total bands for all populations**	**Range of band sizes (bp)**	**Primer with most polymorphism**
OPA set	369	180-3200	OPA-17
OPB set	383	200-3400	OPB-11
OPM set	414	200-3500	OPM-06
HKB17/9	14	260-3200	HKB17/33 + $(GACA)_4$
HKB17/33	13	180-3000	
HKB23/52	16	200-3100	
$(GACA)_4$	13	280-3200	
$(GTG)_5$	11	380-3000	

Cluster analysis using complete linkage and squared Euclidean distance at p=0.05 produced a dendrogram with four clades (orange rectangles) at <30% similarity (**Fig. 3.26**): the first clade contained all populations except 3 and 10, the second only populations 3 and 10, the third all plant hosts and the fourth the two fungi. Principal components analysis also resulted in separation of the populations into distinct groups (**Fig. 3.27**). Populations 3 and 10 were again separated from others, but this time populations 4 and 6 were intermediate between them and the others. The plants and the fungi were widely separated from the galls and grouped as before, except that cauliflower was close to the fungi. Neither type of analysis produced clades that corresponded to ECD types as designated in Chapter 2 (**Table 2.4**), as shown in **Table 3.9**.

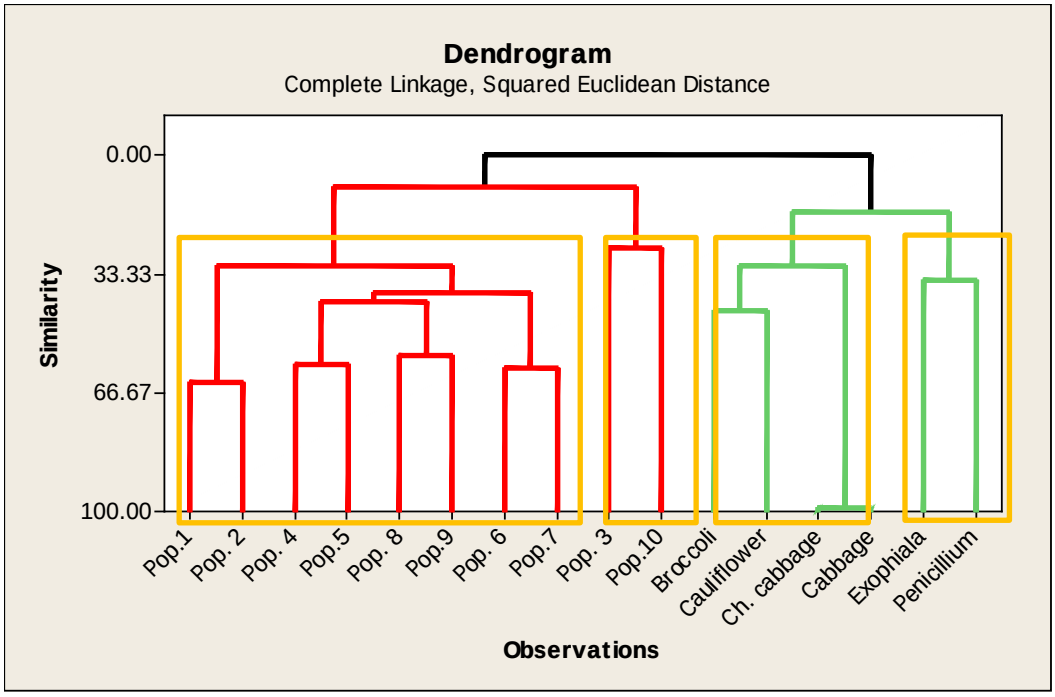

Fig. 3.26. Cluster analysis of RAPD and microsatellite polymorphism in Australian populations using complete linkage and squared Euclidean distance at p=0.05. Orange rectangles indicate (left to right) clades 1-4.

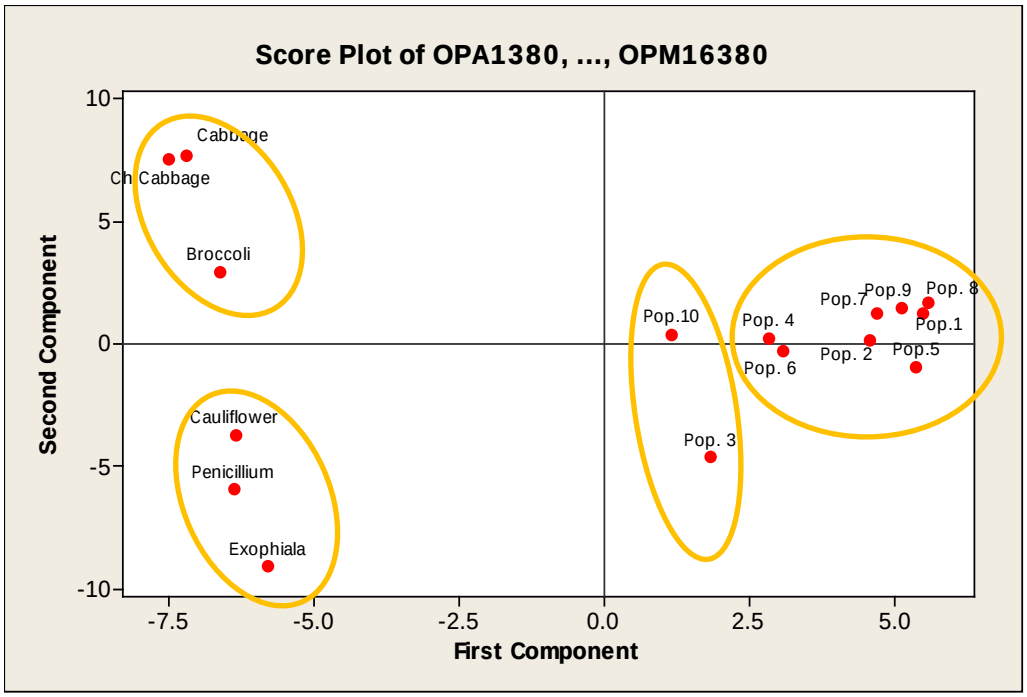

Fig. 3.27. Principal components analysis of RAPD and microsatellite polymorphism in Australian populations. Cabbage and Chinese cabbage overlap. Orange ovals indicate groups 1-4 from the dendrogram (**Fig. 3.26**).

Table 3.11: Comparison of ECD and genetic polymorphism typing.

Clubroot population ID	State of Australia	Property of origin	Date of collection	Host	Previous ECD code	ECD code (this study)	Cluster analysis clade	Principal components group
1	VIC	Boisdale	2002	Cabbage	16/01/31	16/02/15	1	1
2	VIC	Woori Yallock	2009	Broccoli	NA	16/02/14	1	1
3	VIC	Devon Meadows	2008	Broccoli	NA	16/03/15	2	2
4	VIC	Rosebud	2008	Broccoli	16/02/15	16/02/30	1	1
5	VIC	Trentham	1999	Cabbage	16/03/12	16/03/13	1	1
6	VIC	Bunyip	1999	Broccoli	16/02/30	16/02/12	1	1
7	VIC	Werribee	1998	Broccoli	16/03/12	16/03/30	1	1
8	VIC	Lindenow	2008	Cabbage	16/02/31	16/02/31	1	1
9	WA	Manjimup[a]	1998	Broccoli	NA	16/03/14	1	1
10	WA	Manjimup[a]	1999	Cauliflower	NA	16/00/14	2	2

3.4. Discussion

3.4.1 Genetic variation in Australian populations of *Plasmodiophora brassicae*

RAPD and microsatellite primers provided a useful means for investigating polymorphism within populations of *P. brassicae.* There is considerable genetic variation in diversity of populations located within Victoria and Western Australia. Breeding cultivars for resistance in such a genetically diverse population is difficult.

3.4.2 ITS region PCR amplification products ITS-1 and ITS-4

The extraction method devised by Manzanares-Dauleux et al. (2001) produced DNA which was amplifiable with both primers (ITS-1 and ITS-4) as it produced bands of the expected size of approximately 700 bp for all investigated populations (Faggian 2002). However, the ITS primers are non–specific and the bands are not necessarily composed completely of *P. brassicae.* The ITS region was not an useful, efficient or a practical route for studying genetic variation; therefore it is preferable to investigate and work with specific primers (Faggian et al. 1999), as specific primers are more sensitive.

3.4.3 Specific primers to detect *P. brassicae*

The specific primer pairs (PbITS1 and PbITS2) and (PbITS6 and PbITS7) were used for detection of the pathogen in ten collected infected plants (galls). The amplification of *P. brassicae* DNA from these populations with both pairs (PbITS1 and PbITS2) and (PbITS6 and PbITS7) produced single producst of the predicted sizes of around 1100 bp and 620 bp for each pair respectively.

Bryngelsson et al. (1988) and Faggian (2002) list many difficulties faced by researchers seeking to produce a molecular biology-based assay for the detection of pathogens such as *P. brassiace.* These are derived mostly from the soil-borne nature of *P. brassicae* and the difficulties that arise in trying to obtain pure DNA from an obligately biotrophic (non-culturable) pathogen. In soil, resting spores are surrounded by a suite of other microorganisms. Infected plant tissue (root systems) potentially may have the greatest concentration of *P. brassicae* DNA but it is frequently contaminated with host plant debris and microorganisms such as bacteria. Moreover the uptake of random segments of host plant DNA during infection has been reported (Bryngelsson et al. 1988). However, the results reported here show the predicted product and the test provided a negative result when it was used to amplify only host plant DNA against both pairs of these specific primers. Therefore the use of these specific primers as the nested PCR assay is a valid diagnostic test for detection of *P .brassicae* in various environments (water suspensions, infested soil and infected plants). Use of such a test can provide an opportunity for farmers

and seedling producers to determine the source of any contamination by the pathogen in their production system (Faggian 2002).

The use of specific primers for pathogens such as *P. brassicae* can be considered an appropriate assay as they are pathogen-specific and significantly faster than existing (non-molecular) methods, with results being obtained over one day, depending on the quantity of samples needing to be investigated. The process of setting up the assay is relatively simple as it does not require complicated laboratory facilities for the preparation of samples, regardless of whether they originate from plant tissue, water or soil. The nested PCR assay has the potential to form the basis of diagnostic testing for the detection of *P. brassicae*, provided that all new pathotypes of the pathogen can be detected (Buhariwalla et al.,1995a; Manzanares-Dauleux et al. 2001; Rosa et al. 2010).

3.4.4 Molecular markers to track genetic variation in *P. brassicae*

RAPD and microsatellite primers distinguished populations of *P. brassicae*, with microsatellite markers being superior to RAPD for that purpose. The results presented here partially agree with Buhariwalla et al. (1995a), who state that the amplifaction of *P.brassicae* with RAPD primers has proven difficult to interpret as a result of the possibility of interference between *P.brassicae* template DNA with the host DNA. This issue can be overcome in a number of ways. Manzanares-Dauleux et al. (2001) suggested the use of DNase for 3 hours at 37°C when the sample under investigation is a spore suspension. For root galls or infected roots, it is preferable to peel the outer layer of the roots away as indicated in this study.

RAPD and microsatellite primers provide a useful means of investigating polymorphism within populations of *P. brassicae,* and they have been used by a number of different researchers (Buhariwalla et al. 1995a; Manzanares-Dauleux et al. 2001; Rosa et al. 2010).

RAPD assays were generated using a single 10-mer of arbitrary sequence as a primer in the PCR to amplify genomic DNA where the sequence of the DNA is completely unknown. Genomic DNA from different individual galls gives different PCR products, which can lead to the identification of DNA polymorphism and identify different pathotypes. To observe genetic variation, during the reactions the primer can bind to the template DNA at low stringency leading to amplification occurring when the two annealing sites are within an amplifiable distance of each other and are correctly oriented.

The result here shows that there is clear genetic variation between *P. brassicae* populations, as found by Jones et al. (1982a), Buhariwalla et al. (1995a), Osaki et al. (2008a), Manzanares-Dauleux et al. (2001) in France, and Rosa et al. (2010) in Brazil. The result also indicated that the populations of *P. brassicae* in Victoria and Western Australia are highly heterogeneous for both pathotype and DNA pattern. Among the ten field populations investigated, two populations (3 and 10) appeared very different from the other populations. Manzanares-Dauleux et al. (2001) stated that four pathotypes could not be clearly differentiated using the RAPD data. The findings of Osaki et al. (2008a) and Rosa et al. (2010) are similar to those presented here. Both the ECD and RAPD microsatellite analyses identified much heterogeneity in both pathotype and genotype in the different populations investigated.

The findings in Australian collections are similar to those from overseas. There was genetic variation between all ten different populations investigated in the current study. Likewise Manzanares-Dauleux et al. (2001) studying single spore isolates and field populations, reported a degree of global variability. Both types of PCR assay showed a high degree of variation, similar to that found previously amongst French *P. brassicae* collections (Some et al. 1996) and other populations around the world (Crute et al. 1980; Linnasalmi et al. 1991; Voorrips 1995; Kuginuki et al. 1999; Manzanares-Dauleux et al. 2001). Molecular markers identified more genotypes than could be distinguished using differential hosts, with each isolate having a unique molecular genotype. The high level of genetic diversity observed in a very small scale supports the hypothesis that recombination has an important function in the cycle of the pathogen (Manzanares-Dauleux et al. 2001 and Osaki et al. 2008a).

There was no apparent correlation with geography, e.g. population No.9 and population No.10 were from Western Australia but they were in different clades and had different ECD codes (16/03/13 and 16/03/30). The ten different populations were collected from different locations but some were located close to each other, e.g. populations No.1 and No.8 are located less than 60 km apart and yet were separated by both RAPD and microsatellite assays. Populations No. 6 and No. 7 were classified as one pathotype with low levels of virulence, and were collected from different regions up to 110 km apart, but according to RAPD patterns they appeared genetically similar to each other.

The low correlation between pathotype and PCR pattern may be due to the great degree of DNA polymorphism among populations, as shown in both assays (RAPD and microsatellite).

This genetic diversity is consistent with the hypothesis that suggests that the heterogeneity in each population may have affected the pathogenicity results (Tanaka and Ito 2013). The very high amplicon numbers and complex patterns with RAPD in particular would make any relationship hard to see. Many more ECD-characterised populations/isolates would need to be subjected to this analysis in order to find possible molecular markers linked to phenotypic characteristics such as virulence to a particular ECD host. Moller and Harling (1996) similarly demonstrated that out of 40 primers tested, only three gave isolate-specific profiles and one profile corresponded to the ECD race classification of the isolates. 'The evolution of the pathotype seems to be independent of the evolution of neutral DNA markers, as shown for other pathogens' (Chen et al. 1993; Manzanares-Dauleux et al. 2001).

The sensitivity of RAPD-PCR and screening of the whole genome suggests that RAPD analysis is a more useful tool than sequencing the ITS region to show genotypic variation between and within different populations of *P. brassicae*. as RAPD and microsatellite analysis showed genetic diversity between all populations of *P. brassicae* sampled across Australia. Since a large part of the genome can be screened by RAPD analysis, even small genetic variation is disclosed. The common problem with RAPD-PCR is reproducibility of the amplifications and it is essential to have a consistent protocol to obtain reproducible bands. In this study PCR was performed three times using a single thermocycler (G-STORM 2 thermocycler), with most primers showing consistent banding patterns. DNA purity is also a critical factor to obtain consistent results. Low reproducibility may also reflect DNA damage. Three RAPD primers (OPB-20, OPM-17 and OPM-18) failed to produce positive reactions in the study reported here. Yano et al. (1997) similarly reported one negative result in their study of 15 field populations of *P. brassicae* in Japan using 32 primers.

The microsatellite-based SSR analysis performed here contrasts with the RAPD analysis. All of the selected microsatellite primers were interactive with all populations of *P. brassicae*, suggesting that microsatellite analysis may be preferable for studying genetic diversity of *P. brassicae.* Comparing RAPD and microsatellite-based amplifications, the SSR primers produced clear bands with highly amplified DNA fragments. These banding patterns were easier to identify, with optimal annealing temperatures for each primer resulting in the amplifications from the pathogen being more frequent (Buhariwalla et al., 1995a). The majority of the populations reacted strongly with all microsatellite primers and showed distinct clear banding patterns with strong amplification products apart from $(GTG)_5$.

It is important to increase knowledge of how *P. brassicae* continues to diversify and how changes in genetic variation occur in pathogen populations/single spore isolates. This information is essential in the development of cultivars resistant to *P. brassicae* and management practices that maximise the longevity of resistance in these cultivars. Molecular markers that are genotype-specific can be useful for studying how hosts, cultural practices and environment affect the genetic structure of *P. brassicae* populations.

The analysis of the populations of *P. brassicae* from samples collected from infected fields may not give clear results as the population is likely to be composed of a mixture of different genotypes of the pathogen that exist together in the same field. This could be investigated by forming single-spore isolates from these populations and analysing the first galls produced. Attempts to sequence the population DNA should avoid the problems of heterogeneity. If more than one genotype is present in different proportions in the same sample, then any genotype may by chance undergo amplifications first in the early round of the reactions, which provides more target DNA for later rounds and thus tips the balance of the competitive reaction in its favour; if that happened it would mask the presence of the other genotypes.

3.4.5 Summary and Conclusions

Clear genetic polymorphism was detected in Australian *P. brassicae* populations from fresh or frozen galls using RAPD and microsatellite primers, making it possible to compare populations and possibly trace sources of contamination in new outbreaks of clubroot, but the genotype classification did not conform to the ECD classification.

Classifications such as these are only useful if they are stable. In Chapter 2, it was shown that ECD types were not stable and this raises the question as to whether or not the genotypes are stable from crop to crop. This question is explored further in Chapter 4, where successive hosts were inoculated with galls from the preceding host and mixed inocula.

The difficulty of working with populations of mixed genotypes has led some researchers to select out single-spore isolates, where a single spore is used to infect a single plant and then propagated from the galls. These should theoretically be genetically more uniform, but the initial spore is not necessarily homozygous. Even if there is no genetic stability for populations between successive hosts, there should be uniformity or much less instability in single-spore isolates. Therefore in Chapter 5 the stability of single-spore isolates is also studied.

Chapter 4. Changes with plant passage

4.1 Introduction

Considerable genetic variation was observed, using RAPD and microsatellite primers, in populations of *P. brassicae* collected from field locations in Victoria and Western Australia (Chapter 3). A similar result has been reported previously from Japan (Tanaka et al. 2013). RAPD and microsatellite primers were found to be a fast and reliable alternative to the use of the ECD series of plants which compares variation in the pathogen population based on differential phenotypic reactions of a series of host plants. Classification of the pathogen population based on phenotype, molecular methods or otherwise will only be useful if the genotypes are stable. In Chapter 2, it was shown that pathotypes assigned using the ECD series were not stable and this raises the question as to whether or not the genotypes are stable from crop to crop. This question is explored here by sequentially inoculating successive hosts with galls from the preceding host. The effect of mixing inocula was also explored.

4.1.1 Life cycle and genetic variation in *Plasmodiophora brassicae*

P. brassicae has three stages in its life cycle: survival in soil, root hair infection and cortical infection (Kageyama and Asano 2009). Resting spores of *P. brassicae* remain viable in soil for long periods. During the stages of successful infection, these resting spores release primary zoospores, which reach the surface of a root hair and penetrate through the cell wall. These stages are known as the root hair or primary infection stage. Inside root hairs the pathogen develops to the form of primary plasmodia. There are a number of nuclear divisions that occur concurrently in the plasmodia, followed by cleavage into zoosporangia. Subsequently, 4–16 secondary zoospores are formed in each zoosporangium and released into the soil. Secondary zoospores penetrate the cortical tissues of the main roots; this process is known as cortical infection. The second stage of pathogenesis starts with the secondary zoospores. Two zoospores can fuse, resulting in a dikaryotic zoospore, as reviewed by Ingram and Tommerup (1972). It is not known if fusion is necessary for infection of the root cortex to occur, nor whether different mating types of *P. brassicae* exist. However, genetically uniform single-spore isolates can complete the disease cycle, implying that either

fusion of zoospores is not necessary, or that homothallic genotypes of *P. brassicae* exist (Voorrips 1995). In invaded roots cells, the pathogen develops into secondary plasmodia that are associated with cellular hypertrophy, followed by the formation of visible galls in the roots. The plasmodia finally develop into a new generation of resting spores, which are released back into soil as dormant structures as the root galls decompose. Observations of the cortical infection stage suggest that swelling of *P. brassicae*-infected cells and abnormal cell division of *P. brassicae*-infected and adjacent cells induce hypertrophy and that movement of plasmodia by cytoplasmic streaming increases the number of *P. brassicae*-infected cells during cell division.

Field populations of *P. brassicae* exhibit clear differences in pathogenicity. Jones et al. (1982a) demonstrated that field isolates were not genetically uniform, as they found differences between inoculum obtained from different parts of the same gall or from galls of different plants in the same field. While both the primary (ending in zoospore release) and secondary (ending in gall formation) stages in the life history of *P. brassicae* have been well documented, the transitional phase between these stages is unclear, poorly understood and until recently was largely controversial (Donald et al. 2008) .No empirical evidence exists for genetic exchange, but some researchers propose that the genetic variation is due to reorganization of the chromosomes (Fahling et al. (2004; Heo et al. 2009).

Amongst the many doubts and questions regarding sexual reproduction, the existence of a mating type for the fusion of zoospores has yet to be identified (Voorrips 1995). There is currently no clear evidence of chromosomal recombination as a result of sexual reproduction (Fahling et al. 2004) and no research has been conducted regarding the manner in which genetically identical asexual spores propagate stably in host plants. Moreover, the extent to which these factors change the pathogen population over time and the influence of this on the long-term sustainability of the use of resistant cultivars as a disease control strategy is largely unknown.

4.1.2 The stability of pathotypes through generations and inoculation with mixtures of pathotypes

Few studies have examined the effect of a mixture of pathotypes of *P. brassicae.* These have focused on the phenotypes that result from the interaction of the hosts with mixtures of

pathotypes (Voorrips 1996a). There is also a lack of work investigating the possibility of variation in the same generation and progeny of a single spore isolate of *P. brassicae* (Heo et al. 2009). The single spore isolation method has not produced consistently accurate DNA extraction results due to host DNA contamination and low DNA quantities in the collected pathogen (Moller and Harling 1996; Kleweret al. 2001; Graf et al. 2004).

Thus, in this study, two field populations of *P. brassicae* were monitored using RAPD analysis as they cycled through successive generations of host plants to determine if characteristics were maintained genetically over multiple generations. In addition, dual inoculation of host plants with both of the field populations was conducted and RAPD analysis conducted on the root galls produced to study the effect of mixing populations.

4.1.3 Aims

a. To determine if the genetic markers in *P. brassicae* populations are constant in successive generations.
b. To follow what happens to the genetic markers if more than one population is present simultaneously.

4.2 Materials and Methods

4.2.1 Pathogen materials

Two of the ten populations (No. 4 and No. 10) with very different ECD pathotype profiles (16/2/30 and 16/00/14) were used to investigate the effects of generational change and mixing inocula on the genotype of *P. brassicae*. **Figure 4.1** presents a scheme for all stages of this experiment. Seeds of cabbage (*B. oleracea* - Stock ID CA0826 - S&G Seeds) provided by Dr Caroline Donald were sown in 10 cm diameter pots (surface-sterilised using 5% chlorine bleach) filled with sterilised potting mix (three steam cycles at 105ºC for 1 h each). Four lines were prepared: population No.4, population No.10, mixed population “4+10” and uninoculated control. Fifteen pots were used for each population and treatment; in each pot four seeds were sown.

To prevent cross-contamination between populations (4, 10 and the mixture of both), all pots for each treatment were placed in 65-litre plastic black stacking crates and placed in a glasshouse under the same conditions as in Chapter 2 for the ECD tests.

The method of preparing spore suspension for inoculation of host plants was as explained in Chapter 2. Ten days after germination (after the emergence of the initial foliage) each 'treatment' seedling was inoculated by pipetting 1 mL of spore suspension; i.e. population No. 4 (16/02/30), population No. 10 (16/00/14) or an equal mixture of both at the base of its stem (as in previous ECD tests**)**, while 1 mL of sterile MilliQ water was applied to each control seedling.

The plants were evaluated for infection and disease symptoms 10 weeks after inoculation; roots were collected from each treatment, galls were washed with tap water and cleaned of soil and plant debris, placed in plastic bags labelled with their details, and stored at -20°C. DNA was extracted as in Chapter 3. The extracted DNA was stored at -20°C.

4.2.2 Variation analysis through progeny generations

To determine the genetic stability among generations and genetic variation, RAPD and microsatellite analysis was conducted. Population nos. 4 and 10 were used for this study because they were the least and most virulent populations respectively identified from previous work in Chapter 2.

Eight RAPD primers (OPA-08, OPA-11; OPB-03, OPB-04, OPB-12, OPM-03, OPM-09 and OPM-16) and two microsatellite primers [HKB23/52 and $(GACA)_4$] were used. These were chosen as being the most discriminatory among populations in Chapter 3.

Polymorphism of consistent bands was scored manually based on presence (1) or absence (0) of bands of different sizes for each primer and entered manually into an Excel spreadsheet as a similarity matrix along with results from plant hosts and fungi as in Chapter 3. This matrix was used to generate a dendrogram using hierarchical cluster analysis and to perform Principal Components analysis, both at p=0.05, as in Chapter 3.

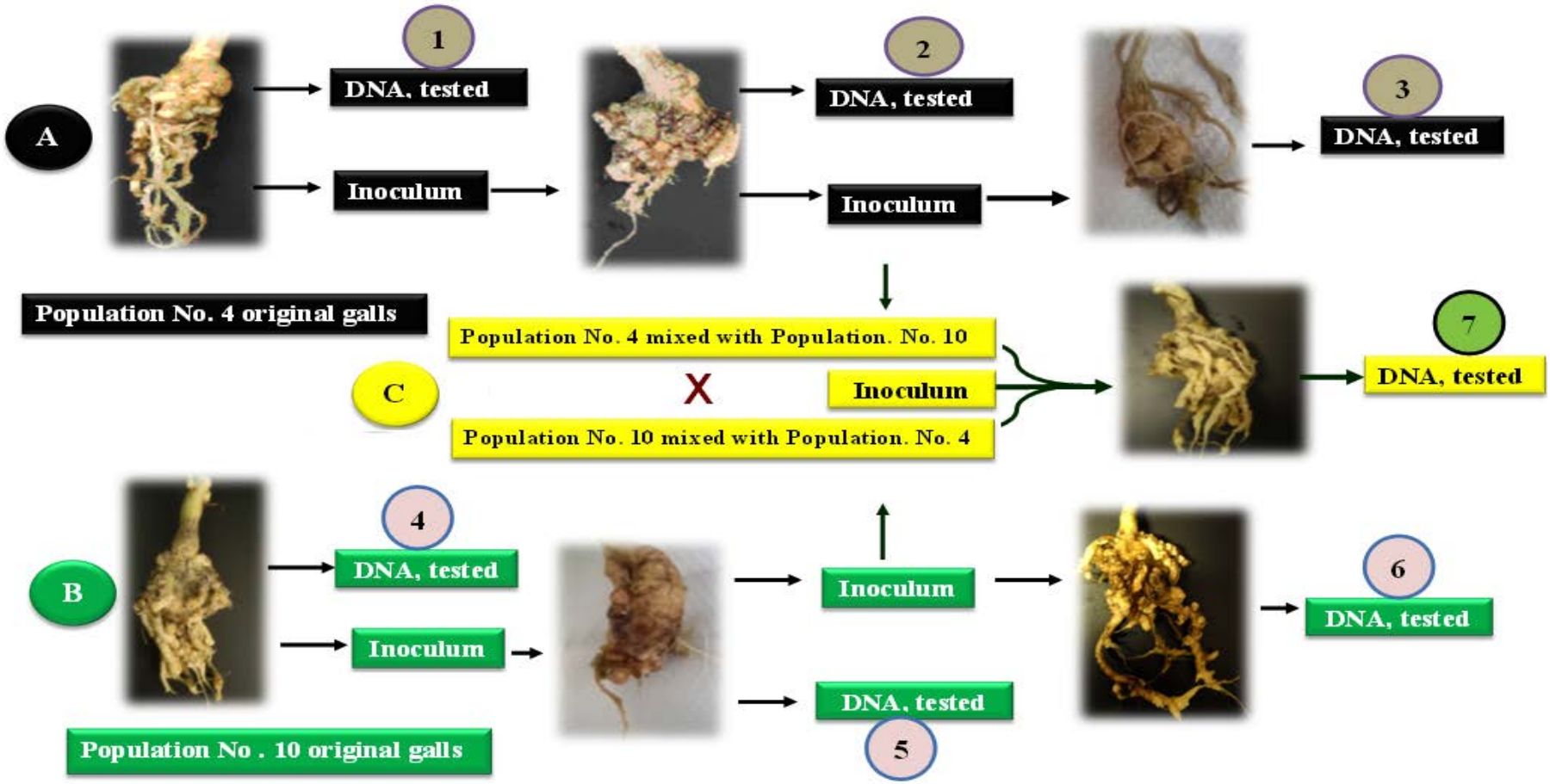

Figure 4.1: Scheme shows the three stages of the experiment for each population. (A). Population 4: 1 = DNA from original galls, 2 = DNA from first generation, 3 = DNA from second generation. (B). Population 10: 4 = DNA from original galls, 5 = DNA from first generation, 6 = DNA from second generation. (C). Mixture: 7 = DNA from galls infected with mixture of populations 4 and 10.

4.3 Results

4.3.1 Detection of symptoms and gall formation

All inoculated plants produced galls; test pathotypes induced clear clubroot symptoms on the susceptible host for all three treatments except control plants, but with varying degrees of gall formation. All target plants were infected, but there was a clear difference between the infected plants, with population No. 4. having smaller root galls compared with the other two treatments (population No.10 and the mixed population"4+10"). Plants infected by population No. 10 had fewer galls than plants infected by mixed populations (No.4 + No. 10) (**Fig. 4.2**). Host plants infected with population No. 4 produced between 22 and 35 single galls on each host plant (**Fig. 4.2a**), while the more virulent population No. 10 produced 90 to 100 single galls on each host plant (**Fig. 4.2b**). The mixed population (No. 4 + No. 10) was the most virulent, producing more than 120 galls per plant, with swelling (hyperplasia/ hypertrophy) evident at the top of the roots (**Fig 4.2c**). No galls were observed in any control host plant (**Fig. 4.2d**). Twenty single galls were collected from each treatment for the purpose of DNA extraction and further investigation.

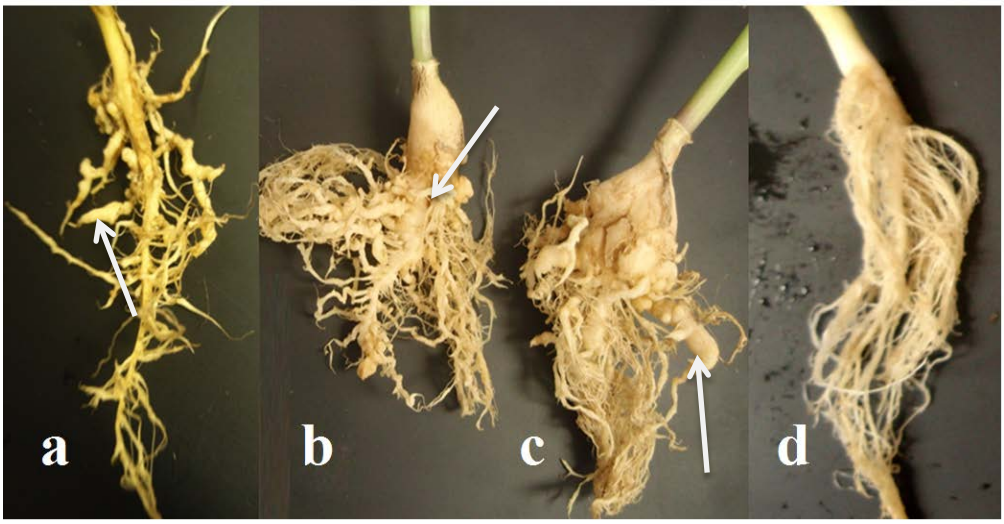

Figure **4.2**: Symptoms and gall formation (arrows) on host plants after inoculation with (a) population No.4; (b) population No.10; (c) mixture of populations (4+10) and (d) control (uninoculated) plants. (arrows point to swelling).

4.3.2 Determination of purity and quantity of genomic DNA

DNA extracted from all galls of the three different populations (No. 4, No.10 and the mixture of No. 4 + No. 10) gave large and acceptable quantities of DNA when run on agarose gel (**Fig. 4.3**).

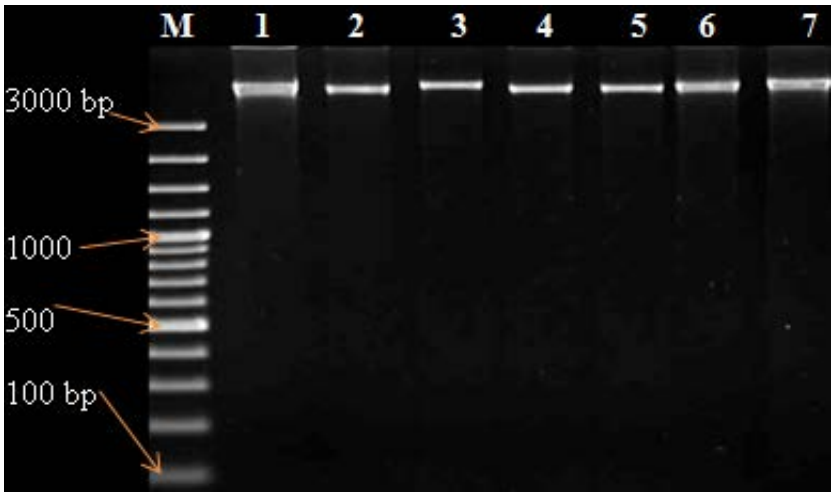

Figure 4.3: Genomic DNA of galls. Lane M molecular weight marker, lanes 1-3 galls from population 4 and two successive generations; lanes 4-6 galls from population 10 and two successive generations, lane 7 gall from a mixture of populations (4 and 10).

4.3.3 Analysis of genetic variation analysis through progeny generations

Analysis of the genetic variation performed using RAPD and microsatellite primers revealed clear variation in the patterns of bands of all populations.

4.3.4 RAPD primers

DNA extracted from galls produced 2-14 polymorphic bands of ~250 to 3200 bp with all RAPD primers (**Figs 4.4-4.5**). All profiles were consistent through replicate PCRs.

DNA profiles were not constant for the same population over successive generations with any of the RAPD primers except for generations 1 and 2 for population 4 with OPA-11 (**Fig. 4.4b**). Each generation produced a unique profile apart from these.

The DNA from the mixed population did not show profiles of both parent populations. A unique profile was produced with each primer, without having much in common with the parent populations (**Figs 4.4-4.5**).

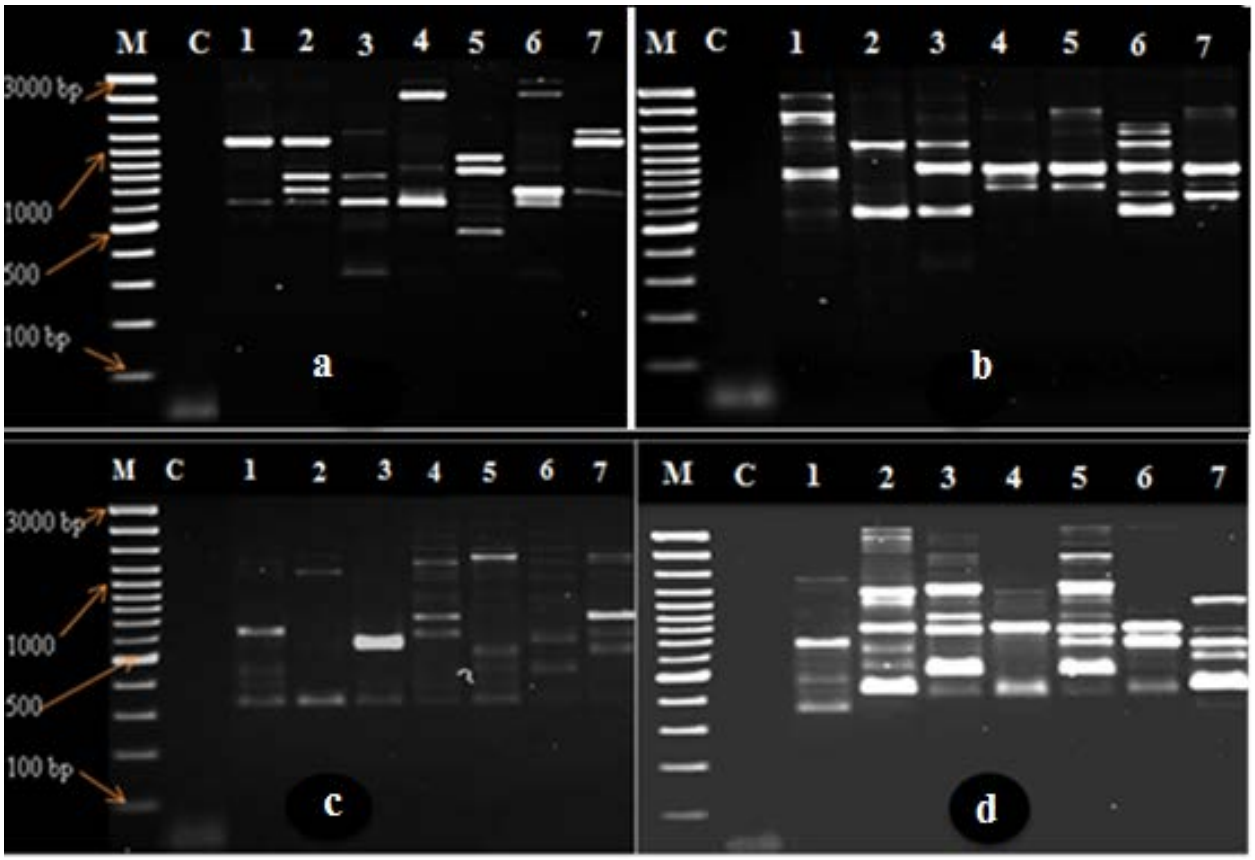

Figure 4.4: RAPD-PCR products from genomic DNA of seven populations of *Plasmodiophora brassicae*. (a) OPA-08, (b) OPA-11, (c) OPB-03, (d) OPB-04. Lane M molecular weight marker, lane C negative control, galls from population 4 (lane 1 original, lane 2 generation 2, lane 3 generation 3), galls from population 10 (lane 4 original, lane 5 generation 2, lane 6 generation 3), lane 7 gall from mixture of populations 4 and 10.

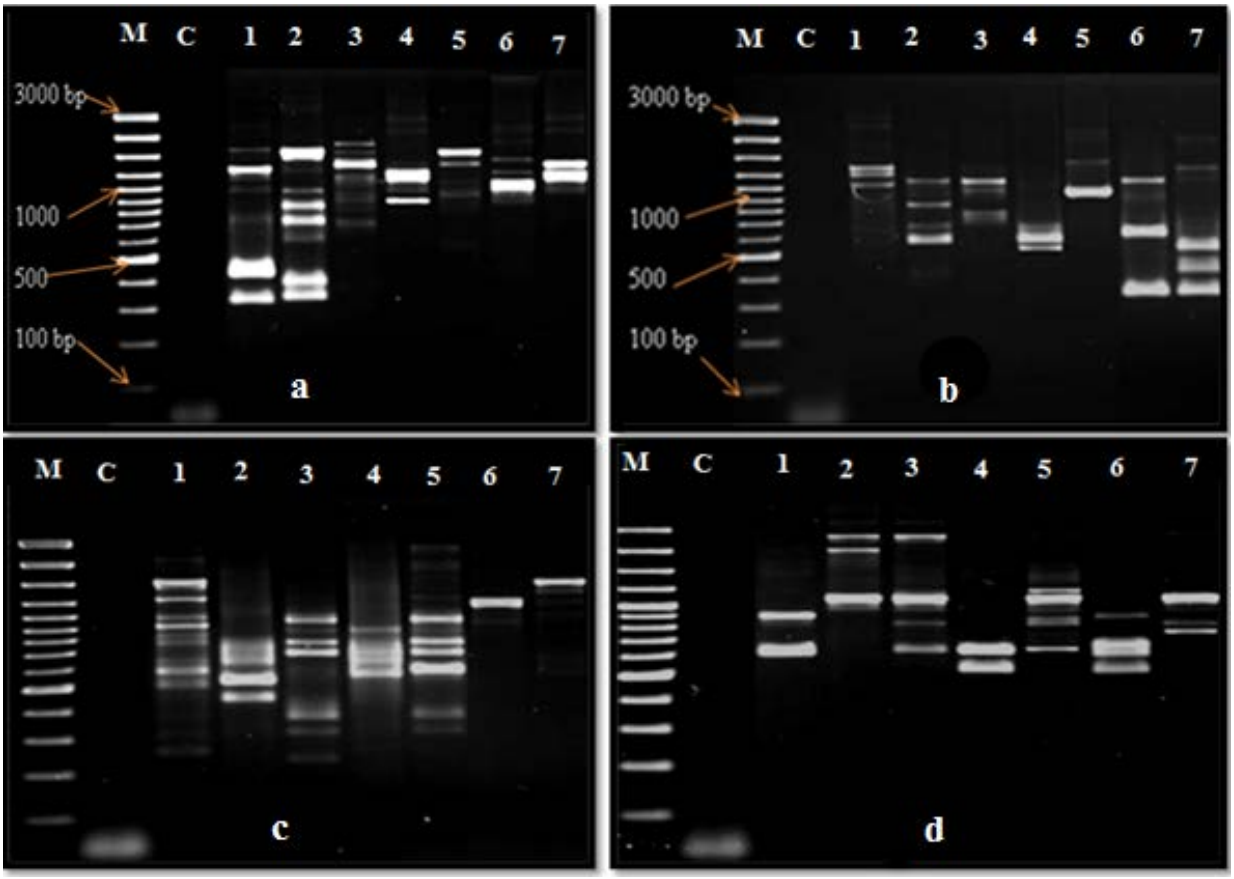

Figure 4.5: RAPD-PCR products from genomic DNA of seven populations of *Plasmodiophora brassicae*. (a) OPB-12, (b) OPM-03, (c) OPM-09, (d) OPM-16. Lane M molecular weight marker, lane C negative control, galls from population 4 (lane 1 original, lane 2 generation 2, lane 3 generation 3), galls from population 10 (lane 4 original, lane 5 generation 2, lane 6 generation 3), lane 7 gall from mixture of populations 4 and 10.

4.3.5 Microsatellite primers

Both microsatellite primers generated reproducible clear bands and showed polymorphism for all populations (**Fig. 4.6**). With HKB 23/52, 1-11 strong consistent bands of ~150-2800 bp were produced (**Fig. 4.6a**), while for $(GACA)_4$ 2-8 bands of ~500-3000 bp were produced (**Fig. 4.6b**).

Successive generations of each of populations 4 and 10 did not produce consistent profiles with either primer, as observed previously with most RAPD primers.

DNA from the gall from the mixture of populations 4 and 10 did not resemble the parents in any generation, as observed with RAPD primers.

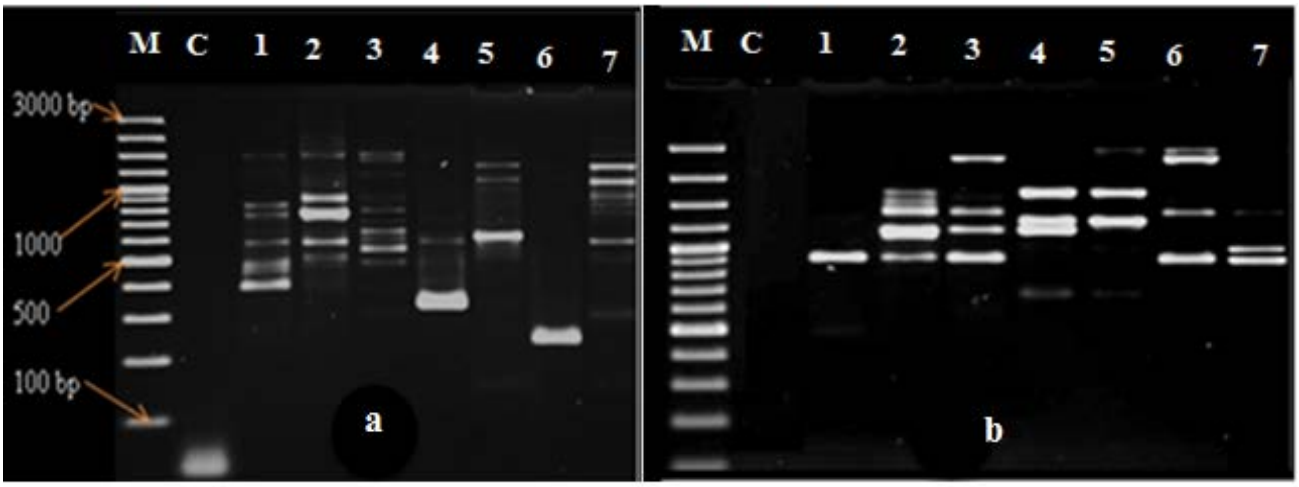

Figure 4.6: Microsatellite-PCR products from genomic DNA of seven populations of *Plasmodiophora brassicae* (a) HKB 23/52, (b) $(GACA)_4$. Lane M molecular weight marker, lane C negative control, galls from population 4 (lane 1 original, lane 2 generation 2, lane 3 generation 3), galls from population 10 (lane 4 original, lane 5 generation 2, lane 6 generation 3), lane 7 gall from mixture of populations 4 and 10.

4.3.6 Cluster analysis

Cluster analysis of DNA from these generational and mixed populations showed 7-35% similarity (**Fig. 4.7**). The generations of population 4 were contained within one clade but the generations of population 10 were not (**Fig. 4.7**). Successive generations were not always most similar to one another. The DNA from the galls from the mixed inoculum was grouped with population 10 and was most similar to generation 3 of this population.

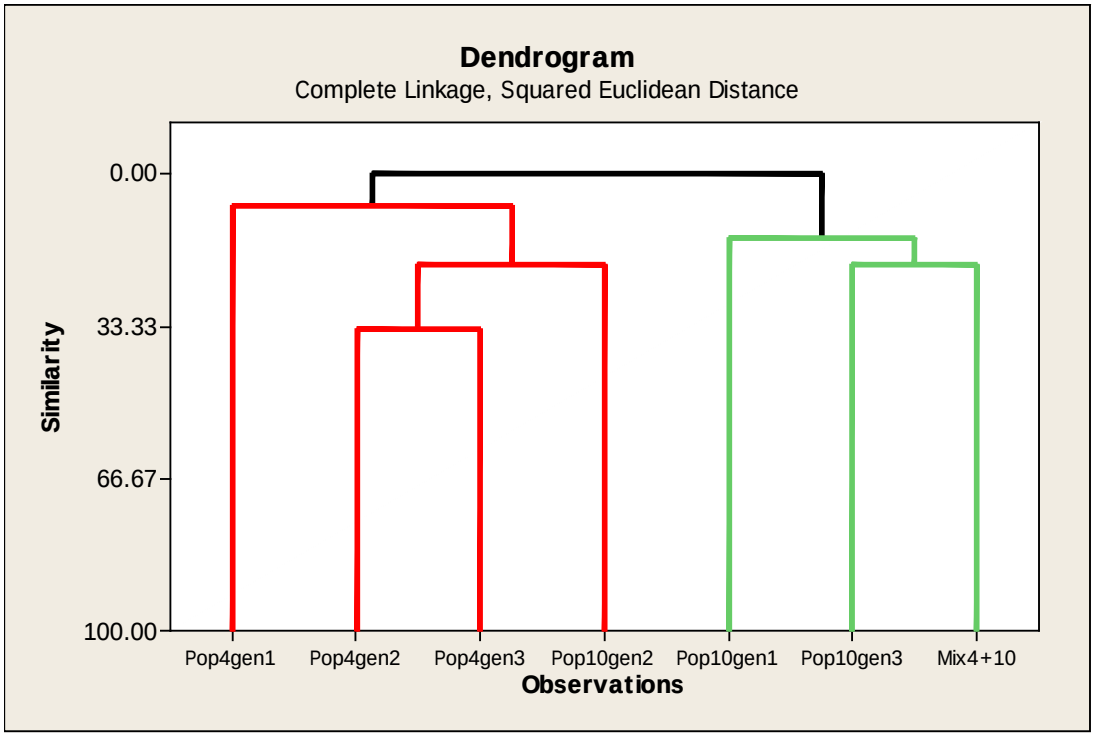

Figure 4.7: Cluster analysis of RAPD and microsatellite polymorphism in DNA from successive generations and mixtures of Australian populations using complete linkage and squared Euclidean distance at p=0.05. Key: Pop4gen1 = population 4, generation 1 (initial inoculum), Pop4gen2 = population 4, generation 2 (galls from the parent inoculum), Pop4gen3 = population 4, generation 3 (galls from generation 2 as inoculum); Pop10gen1 = population 10, generation 1 (initial inoculum), Pop10gen2 = population 10, generation 2 (galls from the parent inoculum), Pop10gen3 = population 10, generation 3 (galls from generation 2 as inoculum); Mix4+10 = galls from mixed simultaneous inoculation using populations 4 and 10.

Principal component analysis of DNA from these generational and mixed populations (**Fig. 4.8**) showed somewhat similar patterns to those seen with hierarchical cluster analysis (**Fig. 4.7**). Generations 2 and 3 of population 4 were clustered closely but were distant from generation 1. Generations 1 and 2 of population 10 were clustered closely but generation 3 was distant. The DNA from the galls from the mixed inoculum was grouped with the four most closely similar generations of both populations.

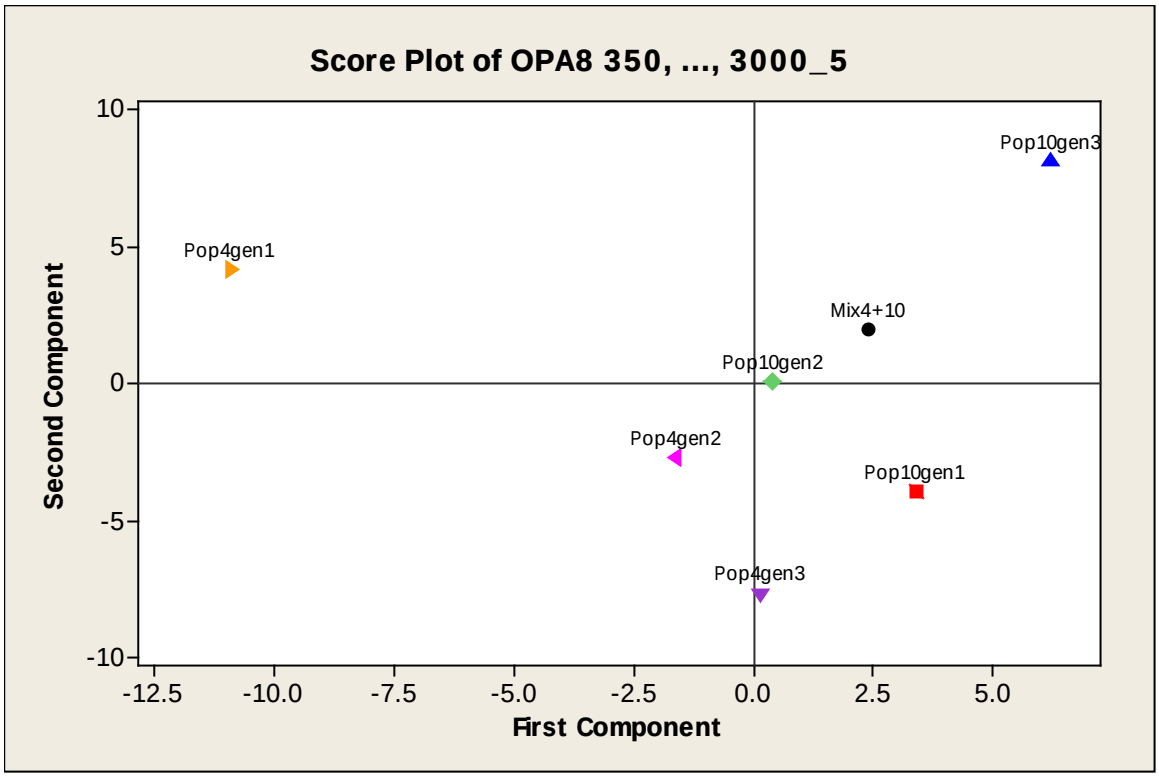

Fig. 4.8: Principal components analysis of RAPD and microsatellite polymorphism in DNA from successive generations and mixtures of Australian populations. Key: Pop4gen1 = population 4, generation 1 (initial inoculum), Pop4gen2 = population 4, generation 2 (galls from the parent inoculum), Pop4gen3 = population 4, generation 3 (galls from generation 2 as inoculum); Pop10gen1 = population 10, generation 1 (initial inoculum), Pop10gen2 = population 10, generation 2 (galls from the parent inoculum), Pop10gen3 = population 10, generation 3 (galls from generation 2 as inoculum); Mix4+10 = galls from mixed simultaneous inoculum of populations 4 and 10.

4.4 Discussion

This is the first study conducted in Australia to investigate the changes that occur in the genotypes of successive generations of *P. brassicae* and when a mixture of two populations is used as the inoculum.

Genotypes were not stable through successive generations, with as much variation between successive generations as between populations, raising the question as to whether or not such typing is useful in control. Mixing genotypes produced unique new genotypes, suggesting that in the field, a genetically diverse *P. brassicae* population produces a diverse population of genotypes in each *Brassica* crop. Breeding cultivars for resistance to such a genetically diverse and dynamic population is intrinsically difficult and targets chosen must be uniform across genotypes.

4.4.1. Stability of genotypes between generations and in mixtures

Successive generations frequently produced different banding patterns. The most likely cause of this observation is that each field population could potentially contain more than one pathotype. During the preparation of inoculum galls were chosen randomly. These could also contain different pathotypes, further complicating the outcome. When DNA was extracted, several galls were used to obtain DNA for testing. This use of several galls from each population to prepare the inoculum and extract DNA would increase the chance of multiple pathotypes being present in the inoculum. Populations of *P. brassicae* are known to be highly heterogeneous for both pathogenicity and DNA banding pattern (Manzanares-Dauleux et al. 2001). To minimise the likelihood of populations containing mixed pathotypes, inoculum should be prepared using only a single small gall.

With the exception of one sample (population 10, generation 2), all generations of the same population fell within the same clade during cluster analysis. Heo et al. (2004) evaluated a group of primers for their effectiveness in pathotype differentiation in *P. brassicae* and showed that only three had a high enough number of bands and differences in banding patterns to separate pathogens according to the pathotypes. In the current study there was no stability in the pathogen genotypes over successive generations but some generations showed some similar patterns with specific primers. For example, generations of pathotype 4 had similar banding patterns when tested with OPA-11 and with OPB-4, and also with HKB

23/52. This could indicate that this population consists of fewer genotypes than the other population studied (No. 10).

The logical result for the expected banding patterns resulting from the mixture of two populations was the appearance of one or both sources of the original populations. However, all banding patterns for the mixture showed the same number or fewer bands than the combined total of the individual populations. A possible explanation for this may be that some parts of the DNA had been destroyed during the extraction process or, as suggested by Fahling et al. (2004) and Heo et al. (2009), specific chromosomal rearrangements associated with particular phenotypes may have occurred, and these may play a role in genetic variability for environmental adaptation.

To date, very few studies have been reported concerning genetic variation within and between field isolates within successive generations or with mixtures of populations (Manzanares-Dauleux et al. 2001). A recent study indicated that each population is often heterogenic and composed of compound genotypes and pathotypes (races) (Tanaka and Ito 2013). This is an important consideration in the breeding of clubroot-resistant cultivars since some field populations may contain small amounts of minor pathogenic genotypes which may be selectively favoured and contribute to the breakdown of clubroot resistance. Moreover, since pathotypes appear not to be stable even through successive generations on the same host species, resistance is also likely to break down gradually as new pathogenic genotypes will be selected for with continual cultivation of CR varieties.

4.4.1.1 Mutation during infection

Mutation is a possible reason for the appearance of new profiles at each generation of resting spores. Most of the studies concerned with mutations in relation to clubroot disease have focused on host mutations that led to resistance for clubroot disease. For example, Devos et al. (2006), Fuchs and Sacristan (1996), Ludwig-Muller (1999a), Kobeltet al. (2000), Grsic-Rausch et al. (2000) and Klewer et al. (2001) have each studied ecotypes and mutant lines of *A. thaliana* as a model host for *P. brassicae* using either disease index (DI) and root index (roots of infected plants/roots of not infected plants (Ri/Rni) susceptible lines “”DI or Ri/Rni.””.

Mutation rates in higher eukaryotes are roughly 0.1–100 per genome per sexual generation but are currently indistinguishable from 1/300 per cell division per effective genome (this

figure excludes the fraction of the genome in which most mutations are neutral) (Drake et al. 1998). Drake et al. (1998) state that mutation rates can rise in microorganisms over short periods due to regulatory or physiological reasons, or more permanently by mutator mutations. Moreover, certain parts of the genome can be maintained inherently hypervariable through specific, local mechanisms such as the cassette switching that mediates phase variation in bacterial and other pathogens and mating type in yeasts and fungi.

The molecular basis determining mutation frequency is unknown. It could be due to differences in DNA-repair enzyme and/or replication proof-reading efficiencies. There is a probability of epigenetic effects of environment. Although high levels of genetic variation in some plant pathogenic fungi have been attributed to parasexuality and/or facilitated gene flow, there is no direct evidence of parasexual recombination. Lamb et al. *(*2008), working with two soil fungi (*Penicillium lanosum* and *Aspergillus niger),* suggested that genetic variation is highly unlikely to be common in relation to mutant phenotypes arising solely by mutation. There is no available information about the mutation rate in *P. brassicae.* It is more likely that the new profiles observed in this study were as a result of chromosomal rearrangement during infection as reported in Fahling et al. (2004).

4.4.1.2 Genetic variation among initial inocula

Hwan et al. (2011a) showed that, with increasing inoculum density, clubroot severity increased and plant height and seed yield decreased. Successful infection and development of symptoms in host plants due to *P. brassicae* requires a high inoculum load. Donald and Porter (2009) and Faggian and Strelkov (2009) state that the number of resting spores required for visual observation of disease symptoms is approximately 1000 spores g^{-1} of dry soil. Jones et al. (1982a) confirmed heterogeneity in a population of *P. brassicae*. Spore suspensions prepared individually from 28 galls collected in a glasshouse showed different reactions. Similarly, Kong Kaw Wa (2009) prepared a clubroot spore suspension from a single frozen gall (weighing >8 g) but several disease indices were reported, suggesting great underlying diversity in such a large gall. An infected field as well as a single gall may be composed of different pathotypes of *P. brassicae* (Donald et al. 2006a; Kong Kaw Wa 2009). Replicate samples of galls from a single site may contain different populations of *P. brassicae* because the distribution of pathotypes in the field may not be uniform.

With such a large number of spores from a pathogen population that is likely to be heterogeneous and to consist of a mixture of different pathotypes, there is a high chance of genetic variation occurring (Xue et al. 2007). Since the populations are likely to consist of a mixture of pathotypes, the spores present will probably differ in virulence, viability and ability to cause the infection. The likelihood that pathotypes within the population will be genetically different is also high, as was observed here.

DNA was extracted from multiple galls for each population in the current study and therefore different banding patterns, representing different pathotypes within the population, might be expected. Likewise the galls used as inoculum may not have been identical to those used for molecular work. Many researchers have used single-spore isolates to eliminate this issue of mixed pathotypes within population of *P. brassicae*. The likelihood of genetic variation could be further reduced by inoculation with just one spore. Trials conducted in this way would be extremely time-consuming and still may not yield the desired outcome since Narisawa et al. (1996) suggested that the likely cause for heterogeneity observed between successive SSI accessions may be due to SSIs not being generated from a single uninucleate resting spore, but from at least one larger spore with 2-4 nuclei.

4.4.2 Stability of genotypes in the presence of multiple genotypes in the field

Information on the composition of pathotypes in the pathogen population and the way in which these interact to affect disease expression is important for the development of *P. brassicae*-resistant host plants and for disease control. If different genotypes are present in the sample, competition between genotypes may mask the presence of a less competitive genotype. Jones et al. (1982a) showed that a pathogenic isolate was inhibited by a non-pathogenic isolate, suggesting competition for infection sites or host resources.

Under field conditions, pathotypes are influenced by the surrounding conditions and thus may be more susceptible to change and diversity. Environmental factors have been identified that influence the selection of those mutations that survive and reproduce (Novak and Brunner 1992). The existence of genetic variation for pathogenicity within field populations is also demonstrated by the response of field isolates to selection pressure (Voorrips 1996a). The major point is that pathogen populations contain mixtures of pathotypes and therefore more variation than expected. This multiplicity and continued appearance of new pathotypes of the pathogen within only one generation will hamper attempts to establish resistant varieties (Cao et al. 2009).

4.4.4 Summary and conclusions

The stability of the number of genotypes with different levels of virulence was investigated through three generations of plant passage by repeatedly inoculating and recovering *P. brassicae*. PCR profiles varied with each generation with all selected primers. Multivariate analysis placed some progeny as most similar to their parent inoculum genotype but others as less than 50% similar. This differs from published studies. Inocula comprising an equal mixture of a low and a high virulence population produced genotype profiles that did not resemble their parent inocula or one another. This was contrary to previous studies in other countries and suggested genetic re-assortment or a very high mutation rate or both. The lack of stability through plant passage poses problems; not only does it call into question the value of genotyping and phenotyping using ECDs but also it makes breeding for resistance to *P. brassicae* very difficult if the amount of polymorphism with microsatellite and RAPD primers reflects that of virulence genes, which is not necessarily the case.

The existence of different pathotypes of *P. brassicae*, i.e. variation in aggressiveness and virulence of isolates, has long been recognised. A means of differentiating isolates and classifying them into physiological races or pathotypes is important in breeding for host resistance and in devising effective control methods. With strains of the pathogen differing in their ability to infect specific host genotypes, strong effort is needed to characterize the virulence of *P. brassicae* and study the populations (Cao et al. 2009) as well as single-spore isolates (Xue et al. 2008) of the pathogen. The extent of diversity within the pathogen populations suggests that existing differential host sets may not reflect the full range of pathogenic diversity in pathogen populations (Howard et al. 2010). A significant challenge in *P. brassicae* research and clubroot disease management has been the absence of rapid and reliable methods for the detection and quantification of the pathogen in soil and biological material. However, the nature of *P. brassicae* as an obligate parasite hampers the application of the majority of techniques for the study of molecular mechanisms of pathogenesis (Hwang et al. 2012b). Further work is required to investigate the cause of the observed diversity, particularly since other researchers have reported that the pathotype composition of *P. brassicae* appeared more diverse when single-spore isolates were examined rather than populations of the pathogen in Canada (Xue et al. 2007).

Since pathogen diversity is so high as to make it difficult to interpret the results of the generational and mixing experiments, a different strategy is needed. Single-spore isolates should reduce or negate the diversity and make interpretation easier.

The aim of Chapter 5 was therefore to investigate the stability of single-spore isolates between accessions and generations.

Chapter 5. Diversity of single spore isolates

5.1 Introduction

Chapter 4 showed that the genotypes of Australian populations of *Plasmodiophora brassicae* changed from generation to generation. Populations of clubroot are heterogeneous by definition and so the change in genotype with generations was not surprising. The genetic variation observed could cause potential problems in trying to breed for resistance to clubroot in local populations of its hosts, although the genetic markers were not necessarily linked to genes important in pathogenicity. This problem could be overcome, as in plant breeding, by starting with a homogeneous population of *P. brassicae* and host plants and using them to establish the relationships between pathogen and host genes, as in Flor's gene-for-gene hypothesis (Agrios, 2005).

Single-spore isolates (SSI) of *P. brassicae* are those believed to be derived from infection of a plant by a single spore (Jones et al. 1982a). Such isolates have commonly been obtained by limiting dilution of a spore suspension as the inoculum for seedlings of *Brassica* species, collection of the gall(s) that form and isolation of the spores inside. They are propagated by using these spores to inoculate *Brassica* seedlings, until sufficient spores have been produced to enable larger-scale studies of pathotypes.

In theory, single-spore isolates from a haploid organism should not vary apart from random mutation. Single-spore isolates are not necessarily homogeneous, but *P. brassicae* is believed (from its life cycle) to be functionally haploid. Using single-spore isolates could thus reduce the amount of genetic variation observed from generation to generation. This chapter therefore focuses on the derivation and use of single-spore isolates to study the amount of variation from generation to generation in *Brassica* species. The brief review below concentrates on single-spore isolation and use, in particular in studying pathotypes and their generational stability.

5.1.1 Derivation of single spore isolates

Several single-spore isolates have been produced in different studies, as summarised in **Table 5.1.**

Early attempts were characterised by low success rates. Buczacki (1977) first described how to obtain single-spores of *P. brassicae.* A suspension containing about 0.1 spores $mL^{-1}$was plated on water-agar. Using a stereoscopic microscope followed by a compound microscope, the spores were located individually on the agar and collected by extracting the fragments of the agar on which they were located. This fragment of culture medium containing a single spore was placed in contact with a 2-day-old seedling in a Germibox chamber and incubated in the dark. After 48 hours, the seedling was transferred to a 200 mL sterile plastic cup containing liquid organic substrate at pH 6.0, and incubated at 20°C with a photoperiod of 12 h. The seedlings were analysed 20 days after inoculation. Galls were collected and stored at -20°C until required. This technique in theory guaranteed that only one spore formed a single gall, but was very time-consuming and the success rate was small; this was ascribed to low spore viability.

Jones et al. (1982a) improved this success rate to 0.7% by using a fine punch attached to a dummy microscope objective to punch out a single spore from the agar medium and by inoculating on to a filter paper over which seedling root hairs had grown on top of moist compost. Scott (1985) doubled the success rate to 1.3% by marking the agar with the objective, removing only larger intact spores with the agar later, covering the inoculated filter paper and roots with moist compost and incubating in the light immediately to improve the health of seedlings. Voorrips (1996a) varied the protocols by using resting spore suspensions instead of agar and achieved 1.2% success rate. Fully developed galls were produced by all methods and Voorrips (1996a) noted that this demonstrated that only one pathotype was needed for successful infection.

Some et al. (1996) varied previous protocols of Manzanares et al. (1994) and achieved a seven-fold greater success rate of 9.6%, an improvement on the 8% reported by Schoeller and Grunewaldt (1986) (cited in Voorrips 1996a). A suspension of 10^4 spores mL^{-1} was spread over 1% agarose, allowed to dry briefly and then the agarose was sliced 1 mm thick, single spores were located and blocks of agar with single spores inoculated on to 4-6-day-old seedlings of Chinese cabbage cv. Granaat in vermiculite. After 24-48 h in the dark, the

Table 5.1: Selected single-spore isolates (SSIs) produced, as reported in different studies, in chronological order.

No. SSIs/plants inoculated (%)	Isolate names	Method of isolation	Author(s)
2/250 (0.8%	Produced clubs from SSI "not reported"	Agar – spread and picked out	Buczacki (1977)
3/450 (0.7%)	1, 2, 3	Agar punch	Jones et al. (1982a)
8/600(1.3%)	A1-A5, B1-B3	Agar marked then picked out	Scott (1985)
2/164 (1.2%)	SSI-1, SSI-2	Droplets of suspension	Voorrips (1996a)
54/561 (9.6%)	17 Msp pathotypes P1(4), P2, P3, P4*(8), P5, P6, P7	Manzanares et al. (1994)	Some et al. (1996)
20/42(46%)	Not reported	Micromanipulation from suspension after gradient centrifugation	Narasiwa *et al*. (1996)
Not reported	SSI 5 (from SCRI 5) SSI21 (from SS 3) SSI 30 (from SS 1)	Not reported	Moller and Harling (1996)
37 from four single clubs	Series M(5), K(9), Pb(18). SJ(5)	Manzanares et al. (1994)	Manzanares-Dauleux et al. (2001)
4/? - not reported	'e' series: e1, e2, e3, e6	Not reported	Schallehn, V., Wagenblatt, B., Siemens, J., Diederichsen, E. (unpublished), in Klewer et al. (2001) and Fahling et al. (2003)
53/1292 (4.1%)	Series M_{36}ES (50 e3, 3 e6)	Manzanares et al. (1994)	Fahling et al. (2004)
24 (4-17%)	Five of each series: SACAN03-1, AbotJE04-1, Leduc-1, ORCA04, CDCN04-1	Droplets of suspension (similar to Voorrips 1996a)	Xue et al. (2008)
2/? – not reported	e4, e9 (=pathotypes 4 and 9 in Williams 1966)	Coverglass method of Kageyama et al.(1995) after sucrose gradient centrifugation of Castlebury et al. (1994)	Heo et al. (2009)

seedlings were replanted in pots of equal parts of steam-sterilised soil and peat. Galls were present when the seedlings were inspected 9-12 weeks after inoculation. Similar levels of infection (4-17%) of plant hosts were recorded by Xue et al. (2008) with five series of SSIs produced from suspensions by a method similar to Voorrips (1996a); suspending the spores in 5% glycerol overcame their tendency to aggregate.

A four-fold increase (46%) in this success rate was claimed by Narasiwa *et al*. (1996) by using a Ficoll type-400 density gradient centrifugation of macerated gall tissue to purify resting spores that were 80% viable as tested by Calcofluor white and ethidium bromide. They noted that 90% of spores were uninucleate but larger spores could be binucleate (10%) or quadrinucleate (0.3%). Single smaller spores were isolated from suspension using an inverted microscope with an automatic system to transfer single cells on to agar in small silicon tubes, to each of which was added sterile soil and a seedling. Seedlings were transferred in the tubes to larger pots and examined at 7 weeks, when they showed only a few small galls; most of these contained only small plasmodia that were thought to be primary rather than secondary and no resting spores were produced. In view of the lack of galls, it is doubtful if the resting spores were functional SSIs, and they would be expected to be uninucleate. This raises the question of the nuclear status of the resting spores used by others that formed galls, especially since Scott (1985) explicitly stated that she used large resting spores. Alternatively the environmental conditions may not have favoured gall production, as the authors commented on the large seedling mortality. The greatest success rate reported so far is the 66% reported by Shulte (1994) (cited in Voorrips 1996a).

The ‘e’ series SSIs have been used widely to study the stability of both genotypes and pathotypes and compare with their parent collections, but their origin is referred to only briefly in Klewer et al. (2001) and Fahling et al. (2004), and the method used to produce them is not clear.

In all cases, the SSIs have been bulked up for experimentation by inoculation of the original galls (sometimes after purification of the resting spores) in a susceptible variety of *Brassica* such as Chinese cabbage cv. Granaat (*B. rapa* L. subsp. *pekinensis*), and either galls or spore suspensions stored at -20°C until required. Neither genotype nor pathotype seems to be checked routinely after this process, though Xue et al. (2008) commented on the greater infection rate from fresh rather than frozen galls.

5.1.2 Uses of single-spore isolates

The establishment and use of lines of most fungi and bacteria from single spores is a common procedure in genetic and pathogenesis studies (Buczacki 1977). This practice is particularly valuable in screening for resistance of host plant breeding material, when the genetic constitution and stability of the pathogen are of considerable relevance (Buczacki 1977).

In theory, SSIs from a haploid organism should not vary apart from random mutation. The purpose in generating them is to assess virulence more accurately in the pathogen and to simplify the process of identifying the genetic basis of pathogenicity and virulence by linking genetic markers to pathotypes. Such isolates have been used to compare genotype and pathotype profiles with one another and with the parent populations from which they are derived. They have also been used to study *P. brassicae* stability from generation to generation.

5.1.2.1 Use of single-spore isolates to study pathotypes and genotypes

Moller and Harling (1996) showed high polymorphism among three unrelated SSIs using 40 RAPD primers (Operon sets A and R); of these, 23 produced amplicons, three produced unique PCR profiles and one (OPA-07) a profile that corresponded to the pathotype. Voorrips (1996a) studied two SSIs generated from the same source and observed no DNA polymorphism or differences in pathotype. He also observed infection by this SSI on hosts resistant to the field isolate, despite the SSI being only a small component of the field isolate, and proposed that the SSI was able to infect because the majority component of the field isolate induced resistance; without this induced resistance, the SSI could infect.

Some et al. (1996) pathotype-tested 17 of the 54 SSIs derived from bulking up the initial galls using seven lines of *B. napus* and two lines of *B. oleracea*, and a disease index was calculated by a modification of that of Williams (1966).

Manzanares-Dauleux et al. (2001) discovered large variation in virulence and DNA polymorphism among 37 SSIs from four clubs in the field in France. There was as much variation in the SSIs from one club in a field as from nine single-gall populations and the variation did not reflect different pathotypes, hosts or geographical origins. A dendrogram generated from 103 loci of DNA polymorphism from 19 RAPD (Operon) and 9 SG primers (Buhariwalla *et al.* 1995) showed 11 groups with 3-70% genetic dissimilarity. The RAPD primers OPL-14 and OPA-13 produced polymorphic profiles that clustered the pathotype P1 (virulent on all hosts) but only at low levels of similarity and, like previous studies, there was no genetic marker linked to pathotype.

Similarly, Klewer et al. (2001) showed that *P. brassicae* sequence-derived primers and RFLP produced polymorphic profiles in SSIs e1, e2, e3 and e6 but that only some RAPD primers were useful in differentiation. SSIs e3 and e6 were genetically closer than to other SSIs although they differed in pathogenicity – e3 stimulated a resistant reaction in two out of four lines of *Arabidopsis thaliana,* whereas e6 did not.

Xue et al. (2008) found that 24 SSIs in Canada had a more diverse range of pathotypes than the parent populations and warned that these cryptic pathotypes could easily become dominant if resistance was bred into hosts only to the dominant pathotypes in populations.

5.1.2.2 Use of single-spore isolates to study stability

Although there have been several studies of the diversity between SSIs derived from the same source, there has been relatively little investigation of their stability over time, an essential prerequisite for their use.

Fahling et al. (2004) mixed two single-spore isolates (e3 and e6) in the inoculum to study genetic variation due to sexual recombination. They found no variation from the parental isolates with electrophoretic karyotyping or RFLP analysis followed by Southern blotting with three repetitive *P. brassicae*-specific probes (H4, E56 and E7) in 53 new SSIs (50 type 3 and 3 type 6) isolated from the single gall formed and so concluded that no sexual recombination occurred. In this analysis, the authors had many distinct bands because of the repetitive nature of the probes and their presence on 15/16 chromosomal bands in *P. brassicae* (Graf et al., 2001, Fahling et al., 2003). Isolate M_{36}ES49 presented the same virulence pattern and fingerprint as e6 by RFLP and Southern blotting but had smaller chromosomes by electrophoretic karyotyping than the parental type; the authors attributed

this to chromosomal rearrangement and proposed that it was probably because of changes in the variable lengths of ‘long tandem repeats’ or ‘tracts of RNA genes’.

In an investigation of genetic stability of SSIs through three generations in South Korea, Heo et al. (2009) found no DNA polymorphism with three primers (URP3, URP6 and OPA-07) in two SSIs and stated that single spore isolates (SSI) of *P. brassicae* were stable genetically following infection. However, the number of primers used was small and testing more may have revealed polymorphism.

Sharma et al. (2013) compared the responses of 84 *A. thaliana* lines to a single-spore isolate of each of pathotypes 3 and 6 (e3 and e6) with those to the corresponding field isolate. Reactions to the single-spore isolates were not strongly correlated with the reaction to the corresponding field isolate, supporting the suggestion of Xue et al. (2008) that the parent populations could hide cryptic pathotypes.

5.1.3 Aims

The aim of this chapter was to investigate the genetic stability through successive accessions and generations of four single-spore isolates (e1, e2, e3 and e6) of *P. brassicae* using RAPD and microsatellite markers selected for their ability to show genetic polymorphism in Australian populations of *P. brassicae* in Chapters 3 and 4.

5.2. Materials and Methods

5.2.1 Pathogen materials

Single-spore isolates (SSI) (e1, e2, e3 and e6) of *P. brassicae* were received in the form of spore suspensions, as shown in **Table 5.2.** Isolates 2, 4, 8 and 10 were generously donated by Prof. Dr Jutta Ludwig-Müller of the Technische Universität Dresden, Germany. They were imported from Germany under a permit to import quarantine material (IP 10002797) from the Australian Quarantine and Inspection Service (AQIS), which did not permit *in planta* use.

Isolates 1, 3, 5 and 9 had previously been imported similarly from the same source in 2006 and used to inoculate plants; isolate 6 was derived from these plants and isolate 7 was derived from plants that had been inoculated with isolate 6. Isolates 6 and 7 were generously donated by Dr Arati Agarwal of the Department of Primary Industries, Knoxfield, Victoria, Australia and were in the form of frozen root galls (derived from infection by single spore isolates). In the following, these isolates are referred to as shown in **Table 5.2**.

5.2.2 DNA extraction

For galls derived from infection by single-spore isolates, spore suspensions were prepared from galls and stored in a small Eppendorf tube at -20°C in a quarantine laboratory until required. Spores were treated according to Manzanares-Dauleux et al. (2001) as before. The spores were dispersed in 100 mM $MgCl_2$, 200 mM Tris pH 7.4 buffer and treated with DNase I (30 µg mL^{-1}) for 3 h at 37ºC to eliminate host DNA. DNA was extracted using a DNeasy Plant Mini-Kit (Qiagen) according to the manufacturer's instructions as in Chapter 3. DNA quality and quantity was estimated by agarose gel electrophoresis followed by staining with ethidium bromide and imaging using a Bio-Rad Gel Doc system as in Chapter 3. For spore suspensions, DNA was extracted from resting spores by the protocol of Manzanares-Dauleux *et al.* (2001). The spores were dispersed in 100 mM $MgCl_2$, 200 mM Tris pH 7.4 buffer and treated with DNase I (30 µg mL^{-1}) for 3 h at 37ºC to eliminate host DNA. The solution was centrifuged at 2500 g for 5 min, and the pellet was resuspended in buffer (5 mM EDTA, 0.5% SDS, 10 mm Tris, pH 7.8) containing 20 mg mL^{-1} proteinase K and incubated for 30 min at 37ºC. After repeated centrifugation, the final pellet was stored at -20ºC.

Table. 5.2: Single-spore isolates of *Plasmodiophora brassicae* and their origins.

Isolate No.	Single-spore isolate (SSI) code	Source	Date obtained/imported
1	e1	Germany (e1a in analyses)	2006
2	e1	Germany (e1b in analyses)	2010
3	e2	Germany (e2a in analyses)	2006
4	e2	Germany (e2b in analyses)	2010
5	e3	Germany (e3a in analyses)	2006
6	e3	DPI, Victoria, Australia (raised in cabbage) (D3a in analyses)	2009
7	e3	DPI, Victoria, Australia (raised in cabbage) from SSI e3 (6) (D3b in analyses)	2009
8	e3	Germany (e3b in analyses)	2010
9	e6	Germany (e6a in analyses)	2006
10	e6	Germany (e6b in analyses)	2010

Numbers from 1-10 refer to different single-spore isolates (SSI) as: 1- e1 old SSI accession, 2- e1 new SSI, 3- e2 old SSI, 4- e2 new SSI, 5- e3 old SSI, 6-e3 SSI generated from isolate 5 in Victoria, Australia, 7- e3 SSI generated from isolate 6 in Victoria, Australia, 8- e3 new SSI, 9- e6 old SSI, 10- e6 new SSI.

DNA was also extracted from seedlings of host plants broccoli, cabbage, cauliflower (*B. oleracea*) and Chinese cabbage (*B. rapa*) and from common soil fungi *Exophiala* sp. and *Penicillium* sp. as these had been isolated from some of the galls and identified by sequencing the ITS region, produced by amplifying their DNA using as in Chapter 3.

5.2.3 PCR

The DNA in extracts was amplified by PCR using primers and conditions as in Chapters 3 and 4. Products were separated by gel electrophoresis and analysed as before.

5.2.3.1 ITS primers

The DNA extracts from the SSI suspensions were tested with universal primers (ITS1 and ITS4) to check for amplicons other than from *P. brassicae*. Any extra bands were sequenced as in Chapter 3.

5.2.3.2 Specific primers (PbITS1/PbITS2) and nested primers (PbITS6/PbITS7)

DNA extracts from the SSI suspensions were tested with specific and nested primers (Faggian et al. 1999), to check that they contained *P. brassicae* DNA as in Chapter 3.2.3.2

5.2.3.3 RAPD and microsatellite primers

DNA extracts from SSIs were assayed by PCR with all sets of RAPD primers (OPA, OPB and OPM) and all microsatellite primers as used in Chapter 3. DNA extracts from plant hosts and fungi were also assayed simultaneously.

Since completing such a lengthy analysis with 60 RAPD and five microsatellite primers was wasteful of time and funds, in view of the high degree of polymorphism found, and the observation that some of the bands from *P. brassicae* overlapped bands produced with DNA extracts from host plants or contaminating fungi, a reduced set of ten primers that still gave good discrimination was chosen to give the greatest discrimination and reproducibility. Polymorphism of consistent bands was scored manually based on presence (1) or absence (0) of bands of different sizes for each primer and entered manually into an Excel spreadsheet as a similarity matrix as in Chapter 3.

5.2.4 Statistical analysis

The similarity matrix of RAPD and microsatellite amplicons was used in Minitab to perform multivariate analyses (Hierarchical Cluster Analysis and Principal Components Analysis), both at p=0.05, as in Chapters 3 and 4.

5.3 Results

5.3.1 DNA quantity and quality

DNA yields from the SSIs were variable in quantity but all were of adequate quality (**Fig. 5.1a**) and comparable to those from the four host plants (broccoli, Chinese cabbage, cabbage and cauliflower) (**Fig. 5.1b**) and fungi (**Fig. 5.1c**).

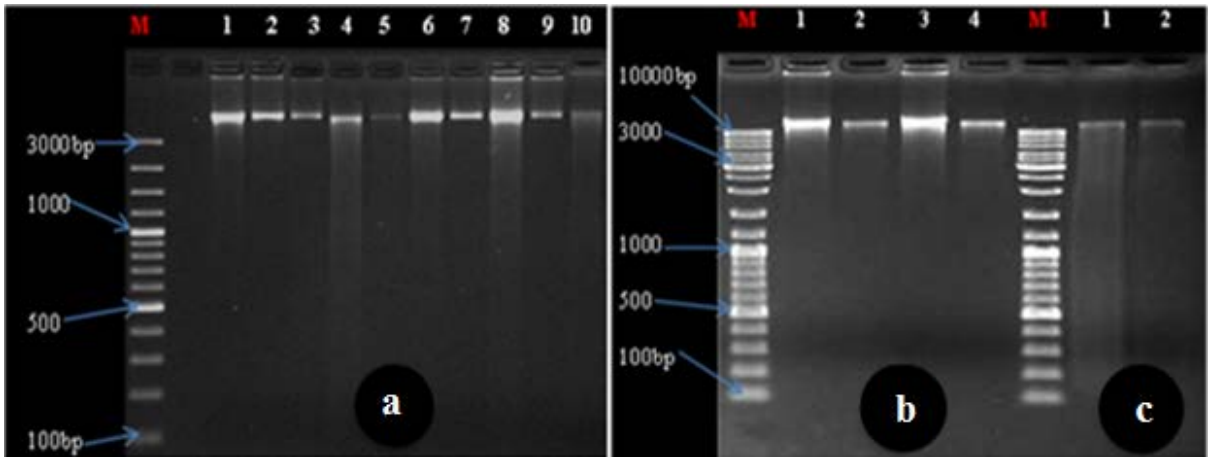

Figure 5.1: Genomic DNA from (a) ten single-spore isolates (SSI) of *Plasmodiophora brassicae*: lane M molecular weight marker, 1- e1 2006SSI, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6-e3 old single spore isolate generated from isolate 5 in Victoria, Australia, 7- e3 new single spore isolate generated from isolate 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate. (b) four clubroot host plants: lane 1 broccoli, lane 2 Chinese cabbage, lane 3 cabbage, lane 4 cauliflower; (c) fungi: lane 1 *Exophiala dermatitidis*, lane 2 *Penicillium chrysogenum.*

5.3.2 ITS primers

Amplicons of ~700 bp were obtained for all SSI samples (yellow box) (**Fig. 5.2**). There were no other bands, particularly of ~800-900 bp (plants) or ~500-600 bp (fungi).

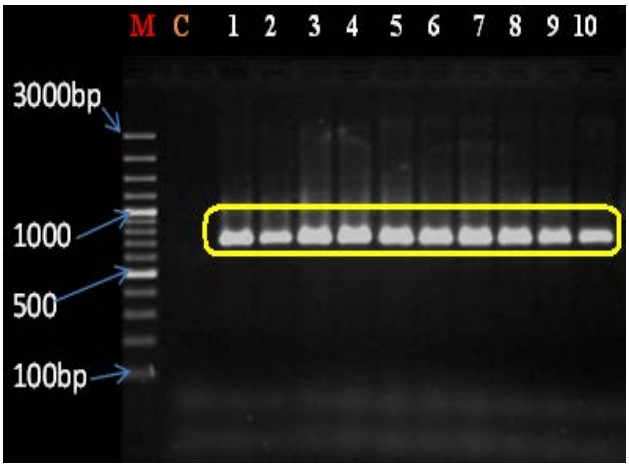

Figure **5.2**: PCR products from amplification of *Plasmodiophora brassicae* SSIs with ITS1 and ITS4 primers. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates:1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated from isolate 5 in Victoria, Australia, 7- e3 new single spore isolate generated from isolate 6 in Victoria, Australia, 8-e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

5.3.3 Specific primers PbITS1 and PbITS2 nested withPbITS6 and PbITS7

Amplification of *P. brassicae* DNA from all SSIs with main primers PbITS1 and PbITS2 resulted in the predicted product size of approximately 1,100 bp (yellow box) (**Fig. 5.3a**). Further amplification of these products with the nested primers PbITS6 and PbITS7 resulted in the predicted size product of about 620 bp (yellow box) (**Fig. 5.3b**).

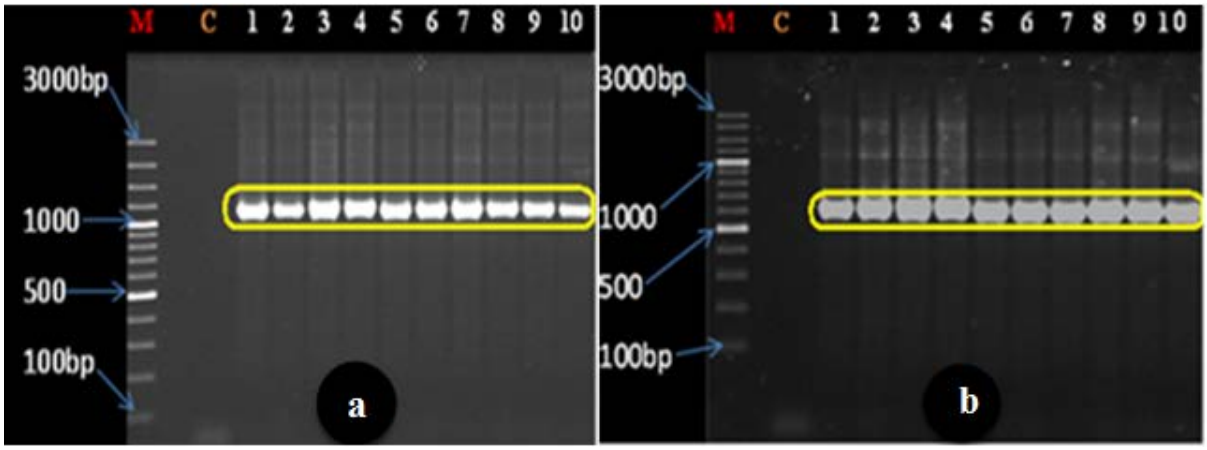

Figure 5.3: PCR products from amplification of *Plasmodiophora brassicae* SSIs with (a) main primers (PbITS1 and PbITS2) and (b) nested primers (PbITS6 and PbITS7. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated from isolate 5 in Victoria, Australia, 7- e3 new single spore isolate generated from isolate 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

The plant and fungal DNA extracts showed no reaction with either pair of primers, as expected (**Fig. 5.4a and 5.4b**).

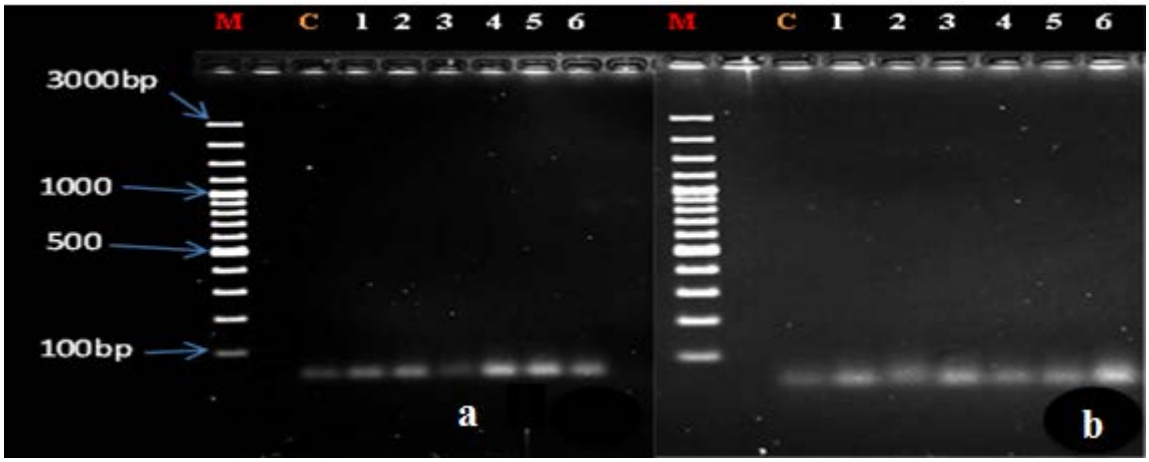

Figure 5.4: Lack of PCR products from amplification of *Plasmodiophora brassicae* SSIs with (a) main primers (PbITS1 and PbITS2) and (b) nested primers (PbITS6 and PbITS7). Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control, lane 1 broccoli, lane 2 Chinese cabbage, lane 3 cabbage, lane 4 cauliflower, lane 5 *Exophiala dermatitidis*, lane 6 *Penicillium chrysogenum.*

5.3.4 RAPD primers with SSI extracts

The RAPD PCRs produced enough information for studies of genetic diversity of single spore isolates of *P. brassicae* (**Tables 5.3-5.4, Figs 5.5-5.18**).

As shown in **Table 5.3**, the three primer sets produced 412-448 bands in PCR. Band sizes ranged from 180-3500 bp and were comparable for each set of primers. The greatest polymorphism with OPA primers were with OPA-01, OPA-02, OPA-08 and OPA-10. With OPB primers, the greatest polymorphism was with OPB-01, OPB-08, OPB-11, OPB-12, OPB-15 and OPB-18. With OPM primers, the greatest polymorphism was with OPM-01, OPM-07, OPM-11, OPM-13 and OPM-18.

Table 5.3: RAPDs analysis results for single spore isolates.

Primers	**Total bands**	**Range of band sizes (bp)**	**Primer with most polymorphism**
OPA set	412	180 – 3600	OPA-10
OPB set	448	180 – 3500	OPB-12
OPM set	431	200 – 3500	OPM-13

Table 5.4: Summary of polymorphism detected using RAPD primers from Operon kits (OPA, OPB and OPM) with single-spore isolates of *Plasmodiophora brassicae*. Key: **+++** strong polymorphism, ++ moderate polymorphism, + weak polymorphism**,** N/A no clear reaction, N/R no reaction.

Primer number	**Primer polymorphism**		
	OPA	**OPB**	**OPM**
1	+++	+++	+++
2	+++	++	++
3	++	+	++
4	+	++	N/R
5	++	++	N/R
6	N/A	++	N/R
7	++	+	+++
8	+++	+++	N/A
9	N/R	++	++
10	++	+	++
11	++	+++	+++
12	++	+++	++
13	++	++	+++
14	N/A	++	N/A
15	++	+++	++
16	++	N/A	++
17	++	N/R	++
18	++	+++	+++
19	++	++	N/R
20	++	++	N/R

5.3.4.1 OPA primers

Primers reacted with all SSI DNA samples. A total of 17 out of 20 OPA primers generated 3-14 clear polymorphic and monomorphic bands ranging from 180-3600 bp patterns (**Figs 5.5-5.9**) for the SSI extracts. Primers OPA-06 and OPA-09 produced no amplicon (**Table 5.4**) and so are not included in the figures. Primer OPA-14 produced indistinct patterns and so is not scored in **Table 5.4**.

Apart from those noted above, all SSIs produced strong consistent polymorphic profiles that indicated clear variation between SSIs imported from Germany in 2006 and 2010 (**Figs 5.5-5.9**). For e3, there was also polymorphism between the profiles for the original imports and those of successive generations (isolates 5-8), e.g. for e1 with OPA-01 (**Fig. 5.5a,** yellow box).

There were also up to four polymorphic genotypes for different generations of the same SSI code, e.g. for e3 with OPA-03 (**Fig. 5.5c,** blue box).

Wide genetic variation between most of the SSIs was evident from the high number of polymorphic markers and unique bands (e.g. **Fig 5.5a, b**, yellow and blue boxes); e3 generations showed clear variation with OPA-01 and OPA-02, each of which produced a unique band for e3b (isolate 6) of about ~800 bp and ~700 bp respectively (**Fig 5.5a, b,** blue box).

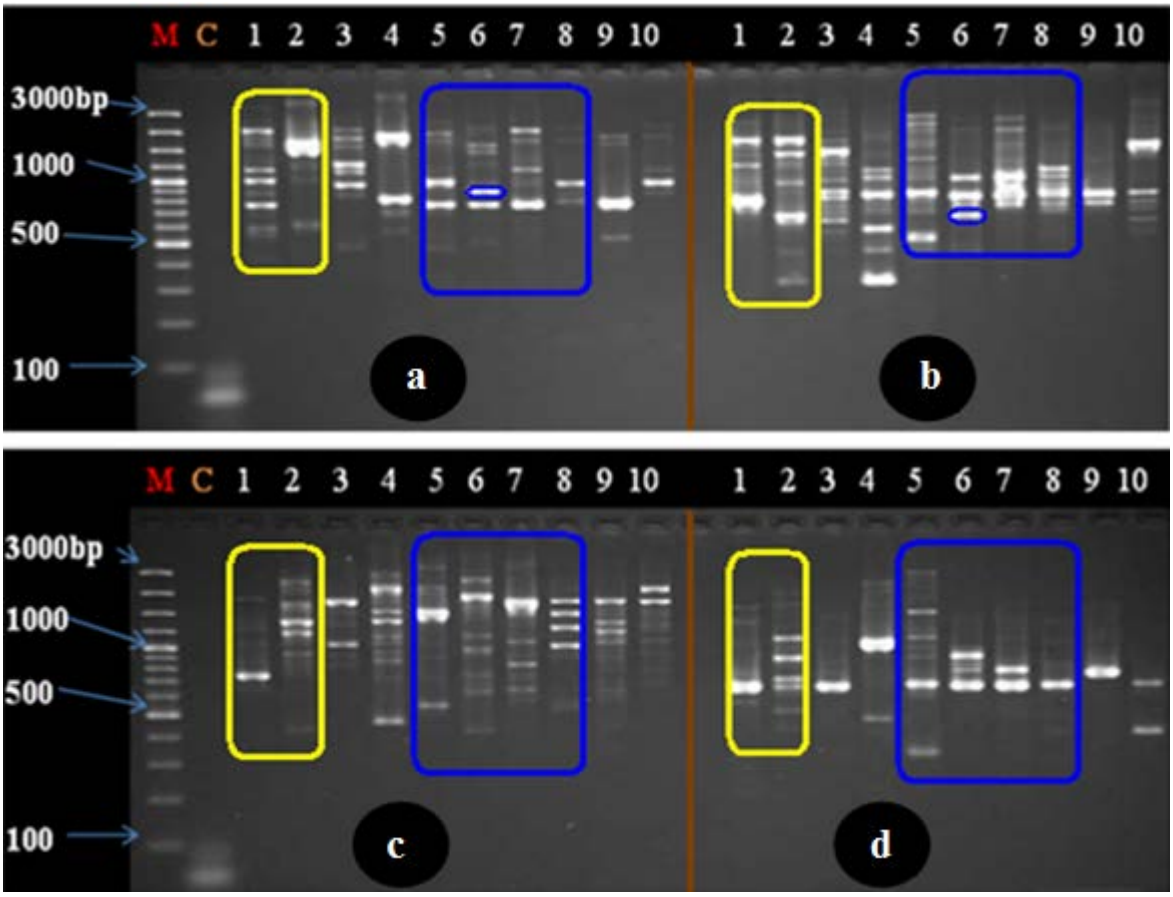

Figure 5.5: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPA-01, b: OPA-02, c: OPA-03 and d: OPA-04. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

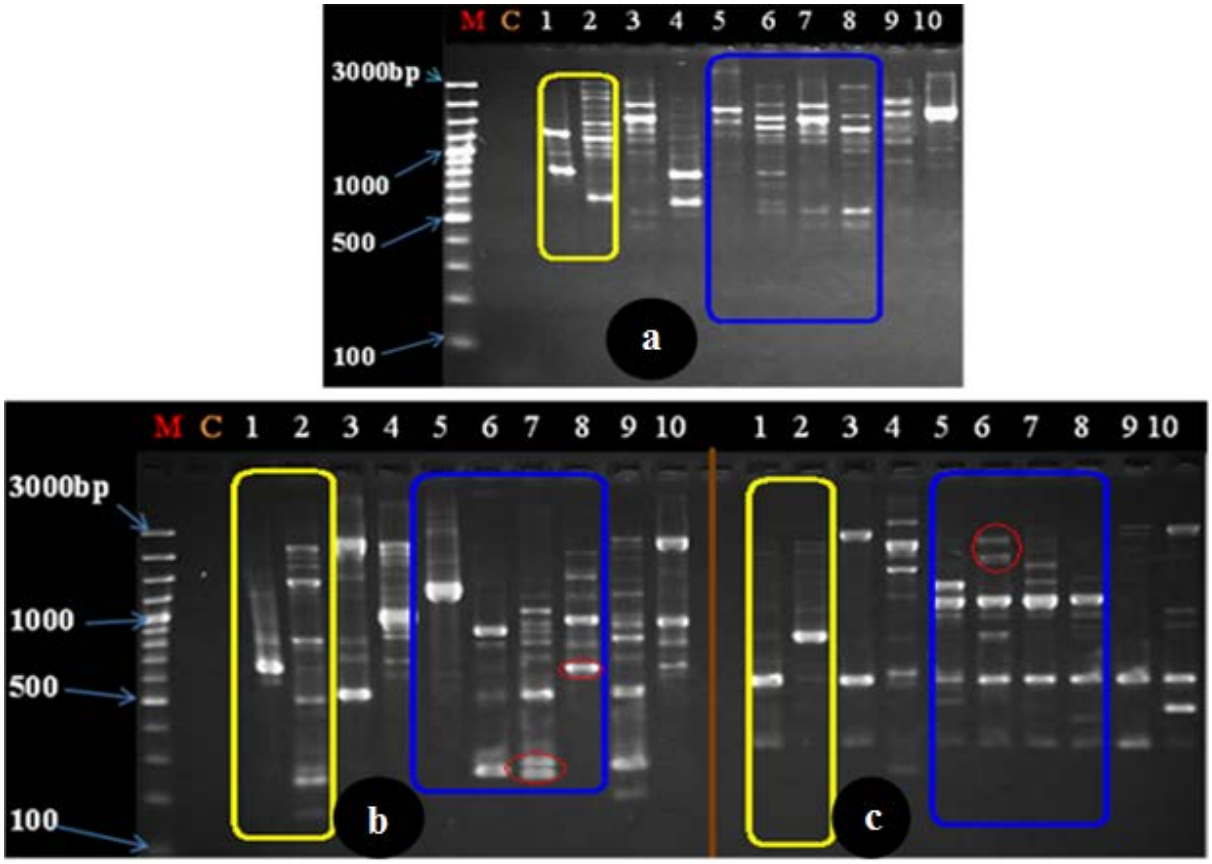

Figure 5.6: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPA-05, b: OPA-07 and c: OPA-08. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

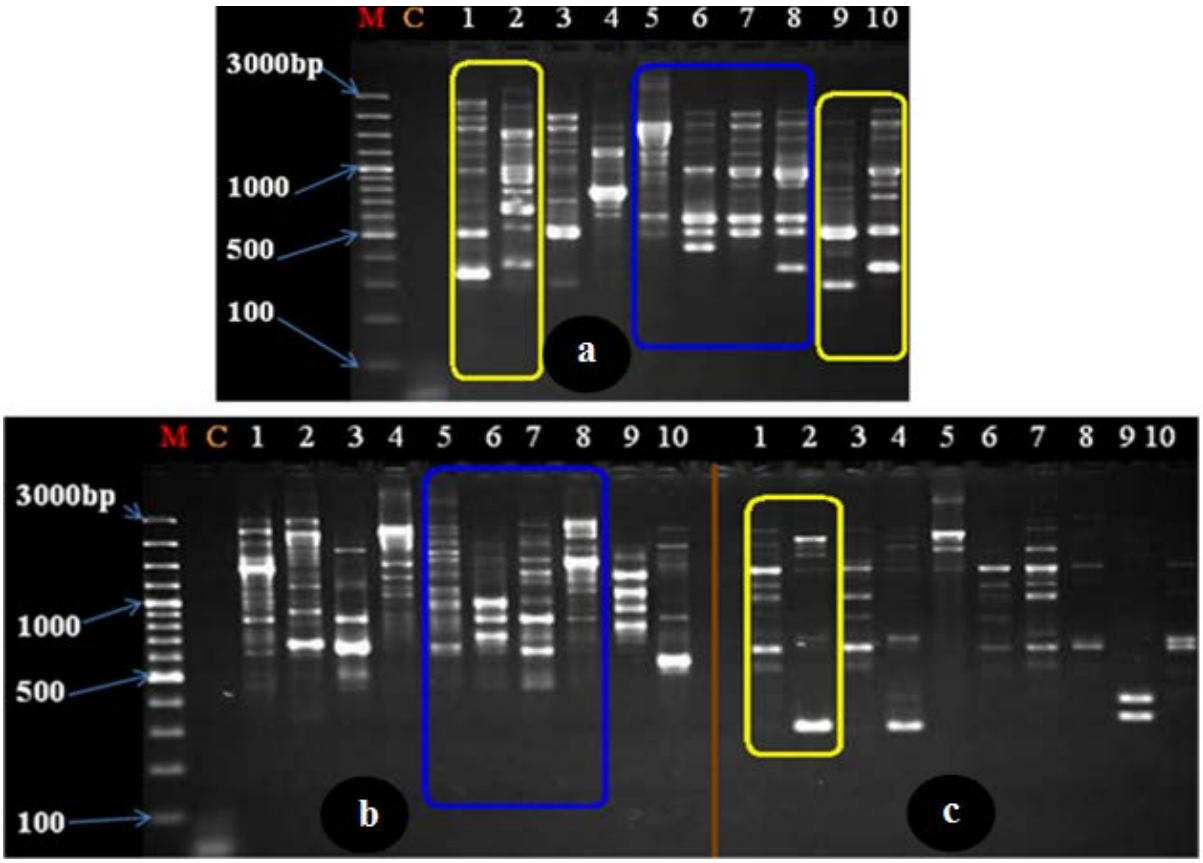

Figure 5.7: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPA-10, b: OPA-11 and c: OPA-12. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

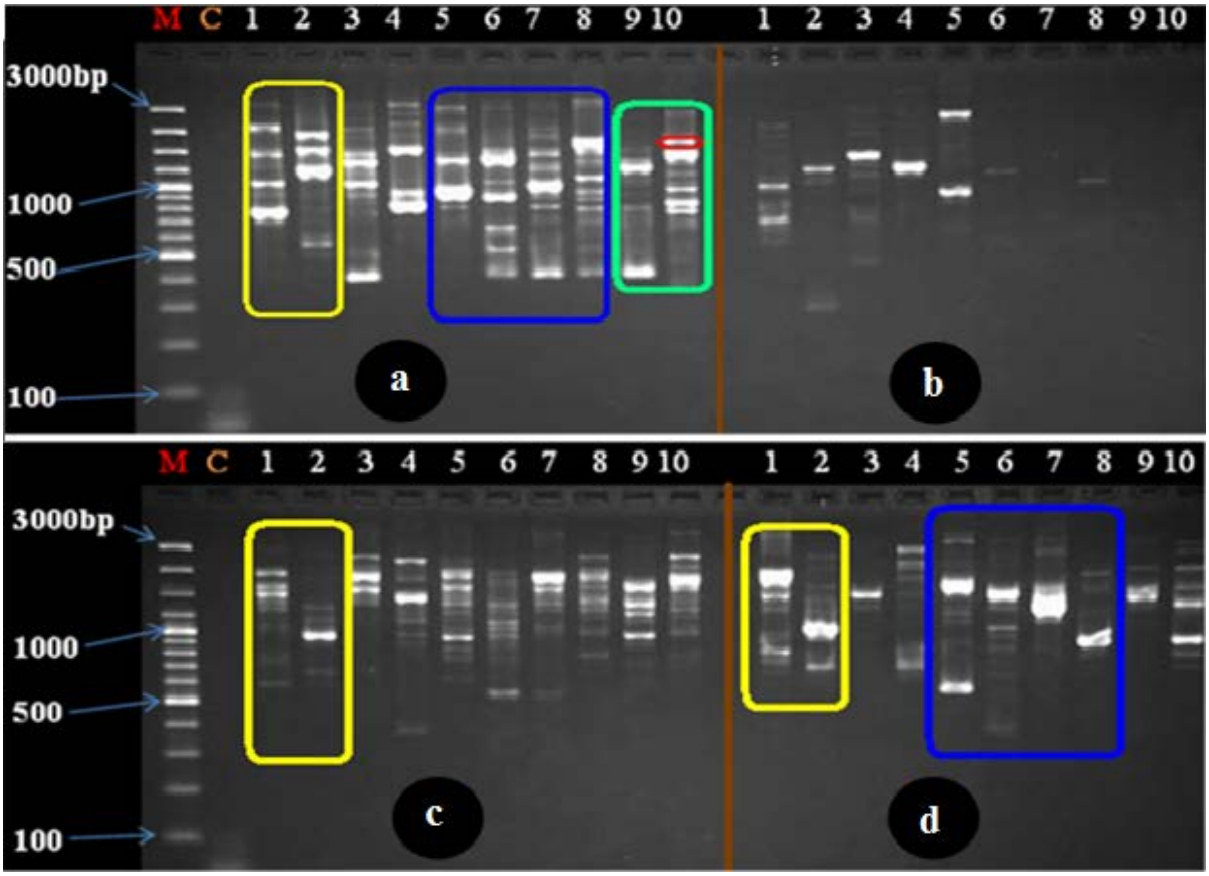

Figure 5.8: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPA-13, b: OPA-14, c: OPA-15 and d: OPA-16. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

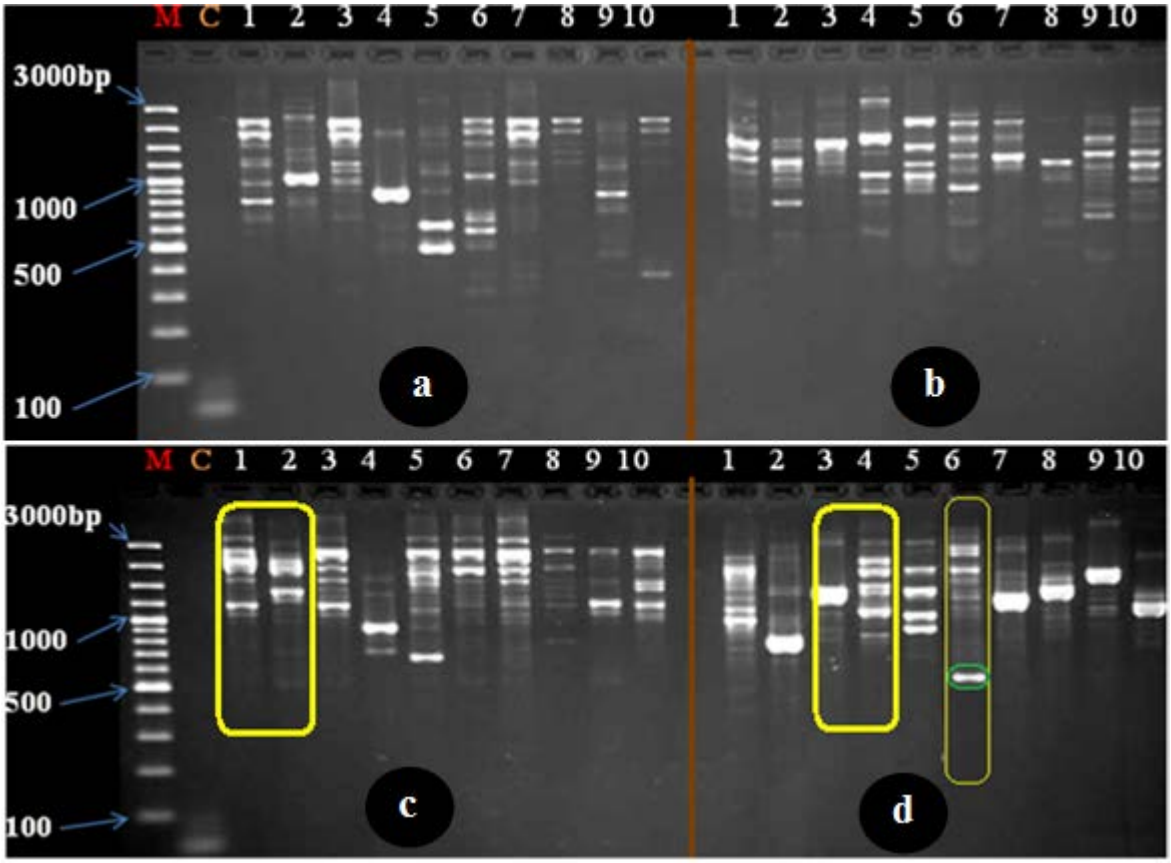

Figure 5.9: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPA-17, b: OPA-18, c: OPA-19and d: OPA-20. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

5.3.4.2 OPB primers

Nineteen out of twenty OPB primers tested amplified 3 to 14 bands and showed good polymorphism among studied isolates. The sizes of the bands obtained were estimated and ranged from 180 to 3500 bp with all DNA extracts (**Tables 5.3-5.4, Figs 5.10-5.14**). Only one primer (OPB-17) did not react with any DNA extract and was recorded as N/R. Also, with OPB-16 no clear reaction was recorded with some samples, and so this has also been recorded as N/A. Most primers produced multiple bands for each extract, producing polymorphic patterns (blue boxes).

The OPB primers that did react indicated clear variation between SSIs imported from Germany in 2006 and 2010, e.g. e1 with OPB-01 (**Fig. 5.10a**, yellow box).

For e3, polymorphic profiles were produced for different generations, e.g. with OPB-01 (**Fig. 5.10**a, blue box), although in some cases there were common bands of the same size, e.g. with OPB-08 (**Fig. 5.11d,** red box).

Primers OPB-01, OPB-08, OPB-11, OPB-12 and OPB-18 showed the greatest polymorphism and number of amplified fragments (**Table 5.4**), e.g. with OPB-2, isolate 2 (e1b) showed a unique profile that was not the same as that of isolate 1 (e1a).

Also, there were up to four polymorphic genotypes for different generations of the same SSI code, e.g for e3 with OPB-01 (**Fig. 5.10a,** blue box**)**, and also with OPB-03. The single spore isolate with code e3 is a typical example that shows polymorphic genotypes with many of OPB primers used.

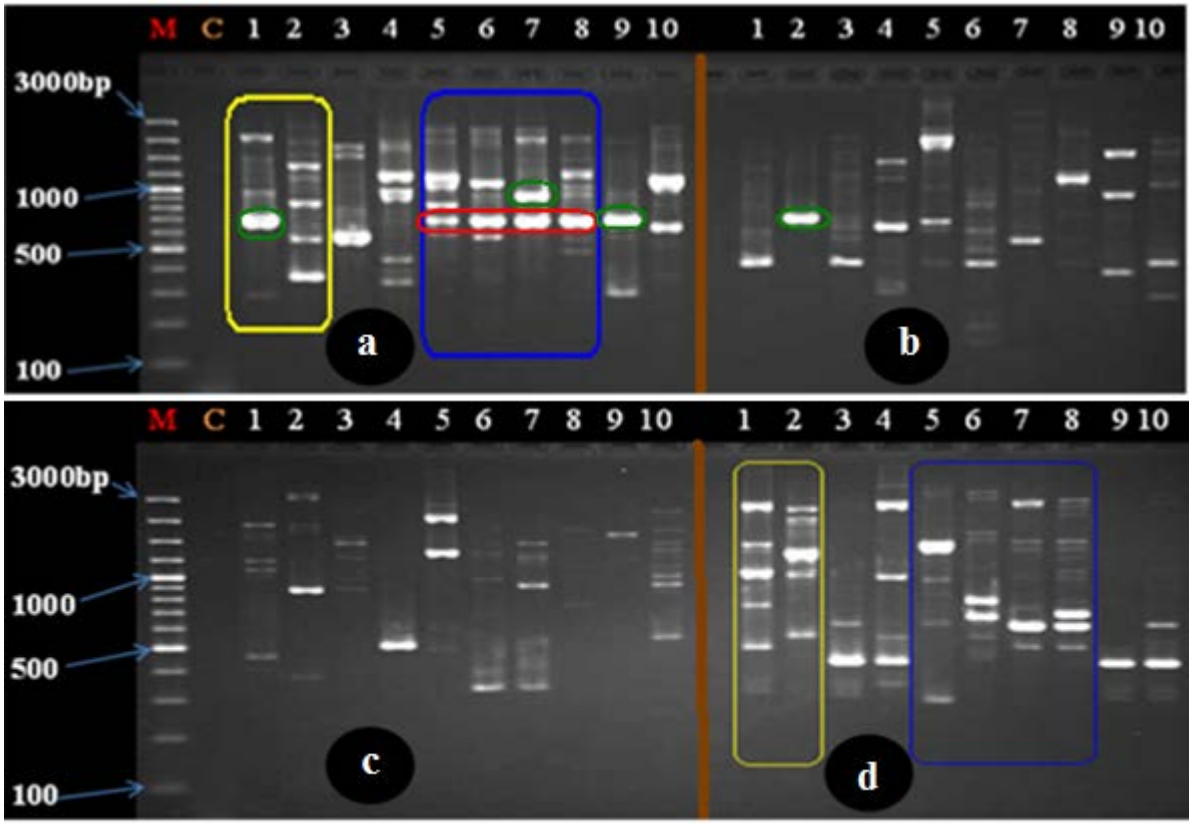

Figure 5.10: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae.* a: OPB-01, b: OPB-02, c: OPB-03and d: OPB-04. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

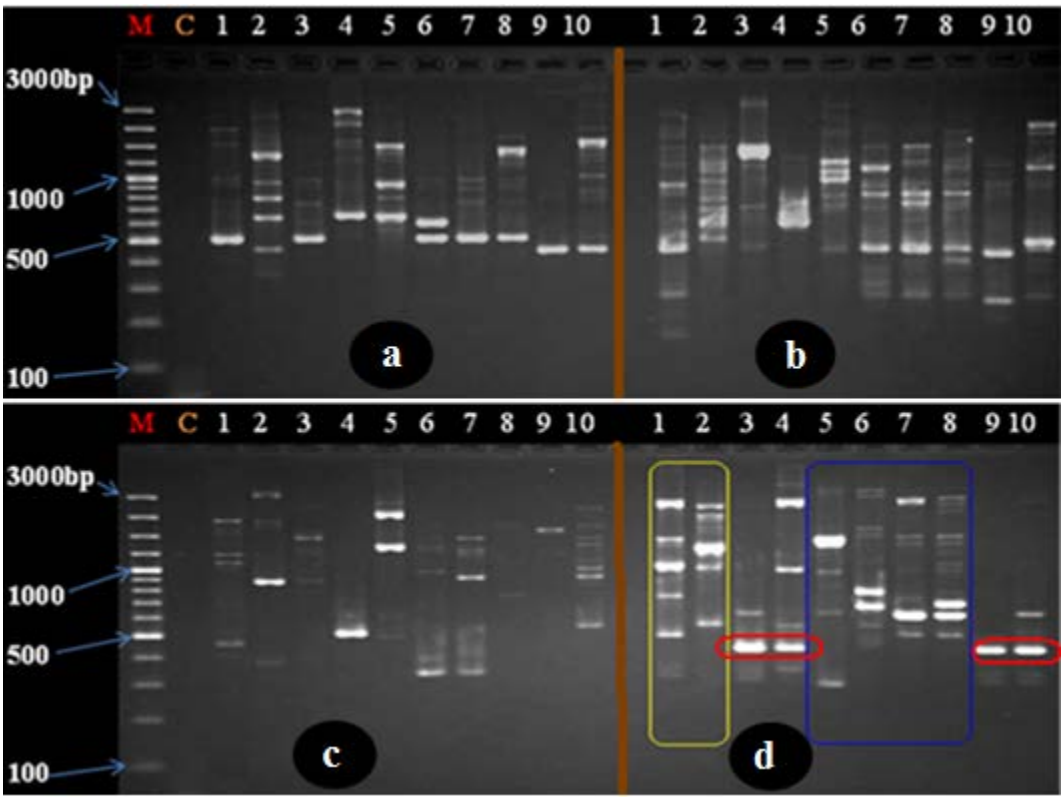

Figure 5.11: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPB-05, b: OPB-06, c: OPB-07and d: OPB-08. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

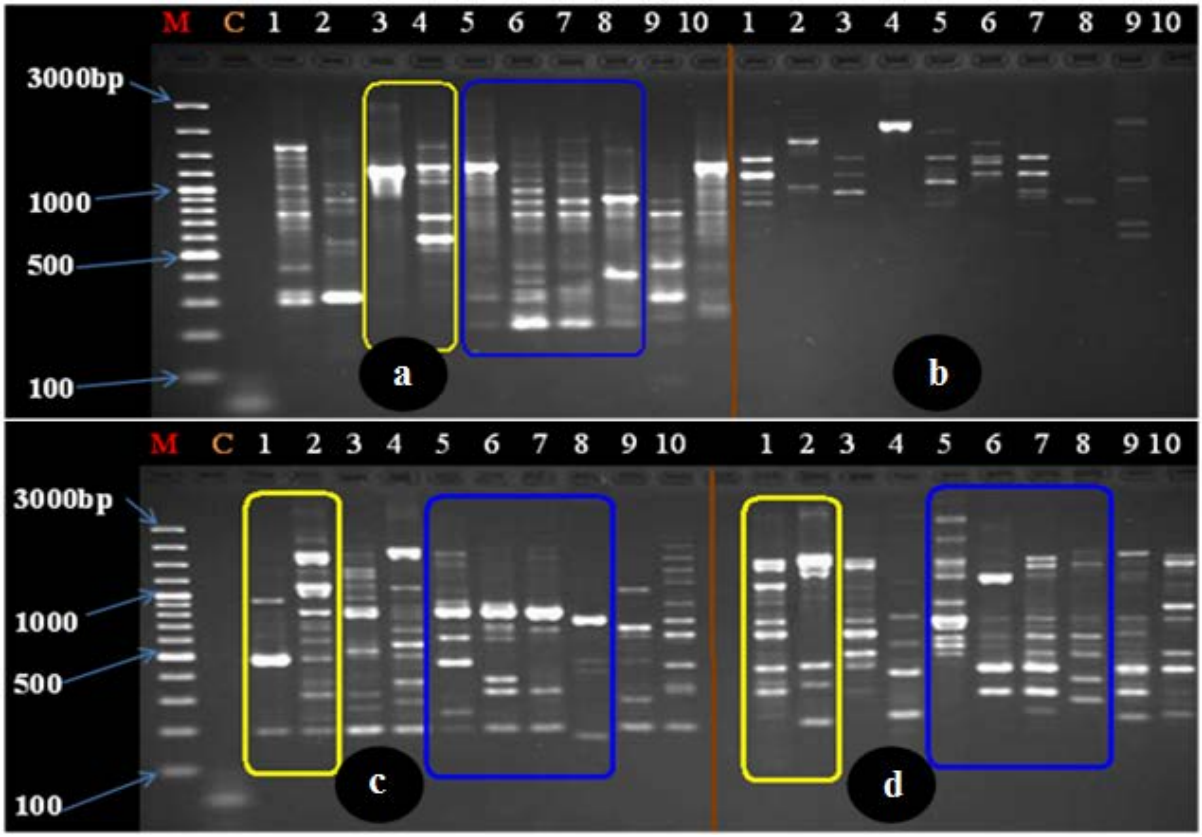

Figure 5.12: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPB-09, b: OPB-10, c: OPB-11and d: OPB-12. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

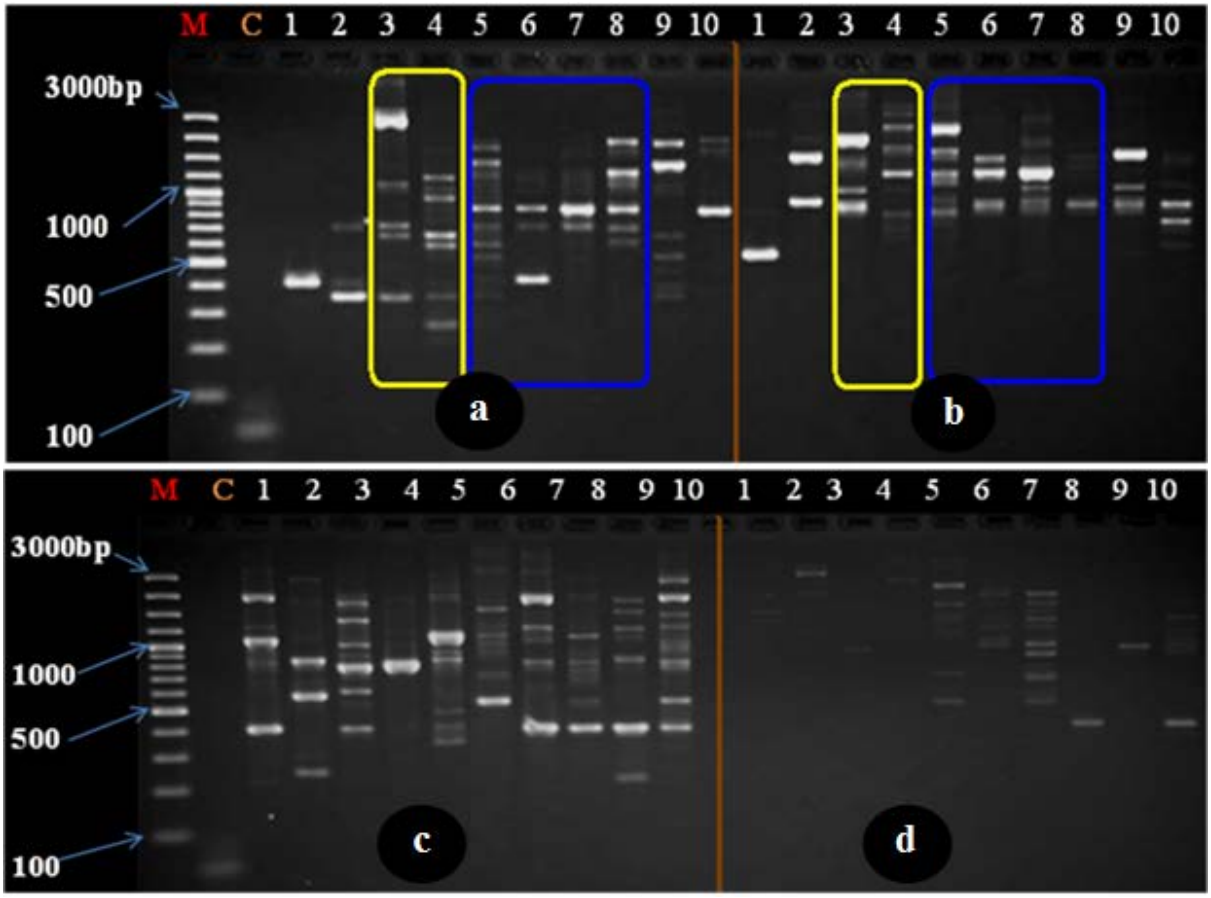

Figure 5.13: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPB-13, b: OPB-14, c: OPB-15and d: OPB-16. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

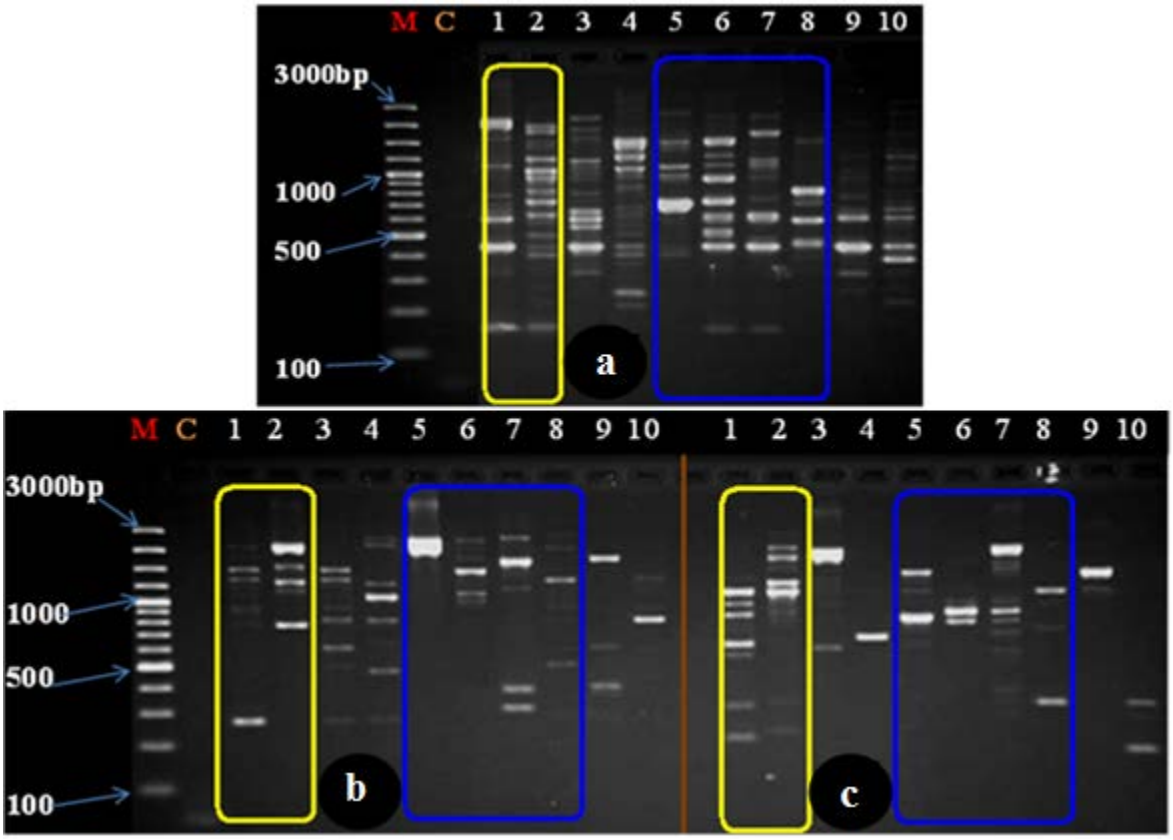

Figure 5.14: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPB-18, b: OPB-19 and c: OPB-20. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

5.3.4.3 OPM primers

Thirteen out of 20 primers reacted with all DNA templates for all SSIs and produced 3 to 14 bands ranging from 1200 bp to 3500 bp, showing clear polymorphic amplifications of DNA samples (**Tables 5.3-5.4, Figs 5.15-5.18**). Primers OPM-04, OPM-05, OPM-06, OPM-19 and OPM-20 did not react and their gels are not shown. Primers OPM-08 and OPM-14 produced indistinct bands (**Figs 5.16b, 5.17d**).

Polymorphism with OPM primers indicated clear variations between SSIs imported from Germany in 2006 and 2010, e.g. e1 with OPM-03 (**Fig. 5.15c**, yellow box). Some band sizes were common to several isolate extracts, e.g. at ~400 bp with OPM-03 (**Fig. 5.15c**, red box).

For e3, different generations and acquisitions again exhibited polymorphism, e.g. with OPM-03 (Fig. **5.15c**, blue box).

Investigation of a wide range of different generations showed clear polymorphic genotypes for the same code of single spore isolates, e.g. for e3. There were also up to four polymorphic genotypes for different generations of the same SSI code, e.g. for e3 with OPM-01 (**Fig. 5.15a,** blue box), and the same was found with OPM-09 (**Fig. 5.16c,** blue box) for the same SSI code e3.

Isolate No.5 (e3a) also showed also a unique band of ~1200bp when tested with OPB-02, which indicated a monomorphic genotype (**Fig. 5.15b** green box).

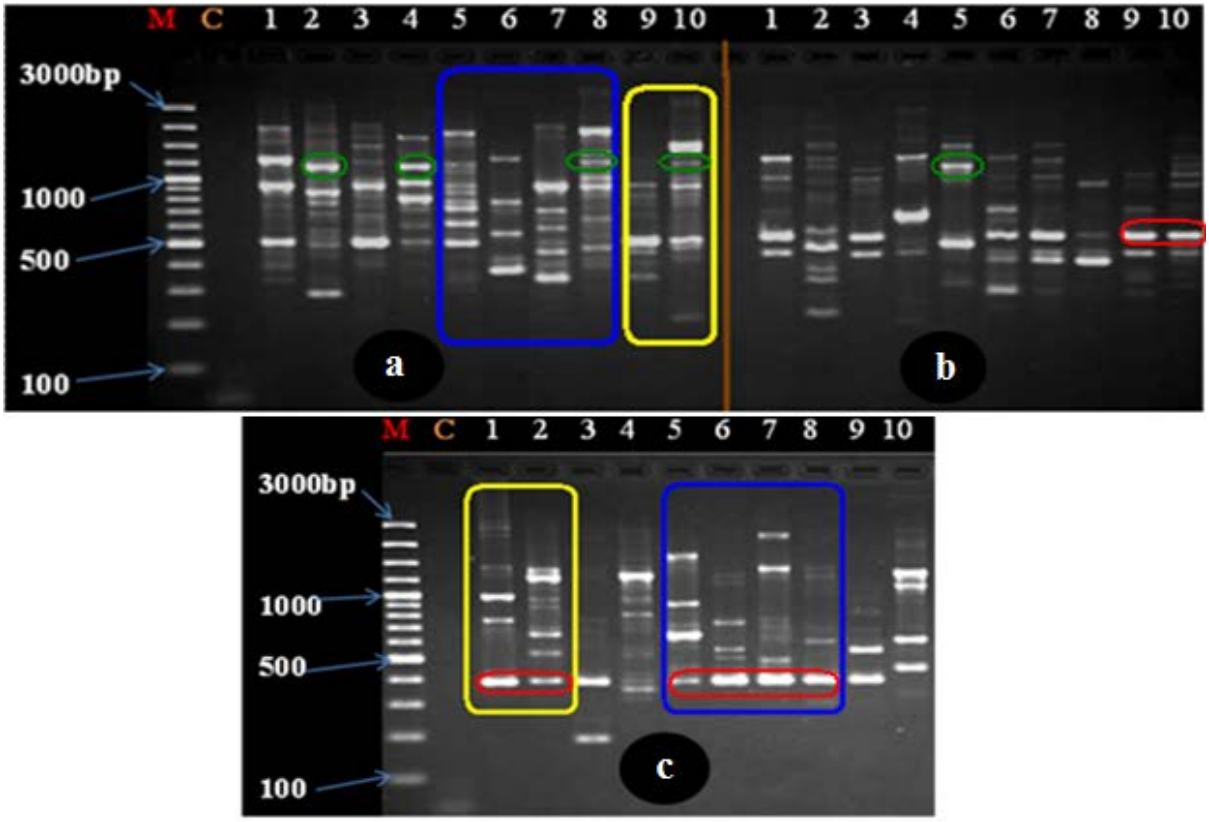

Figure 5.15: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPM-01, b: OPM-02 and c: OPM-03. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

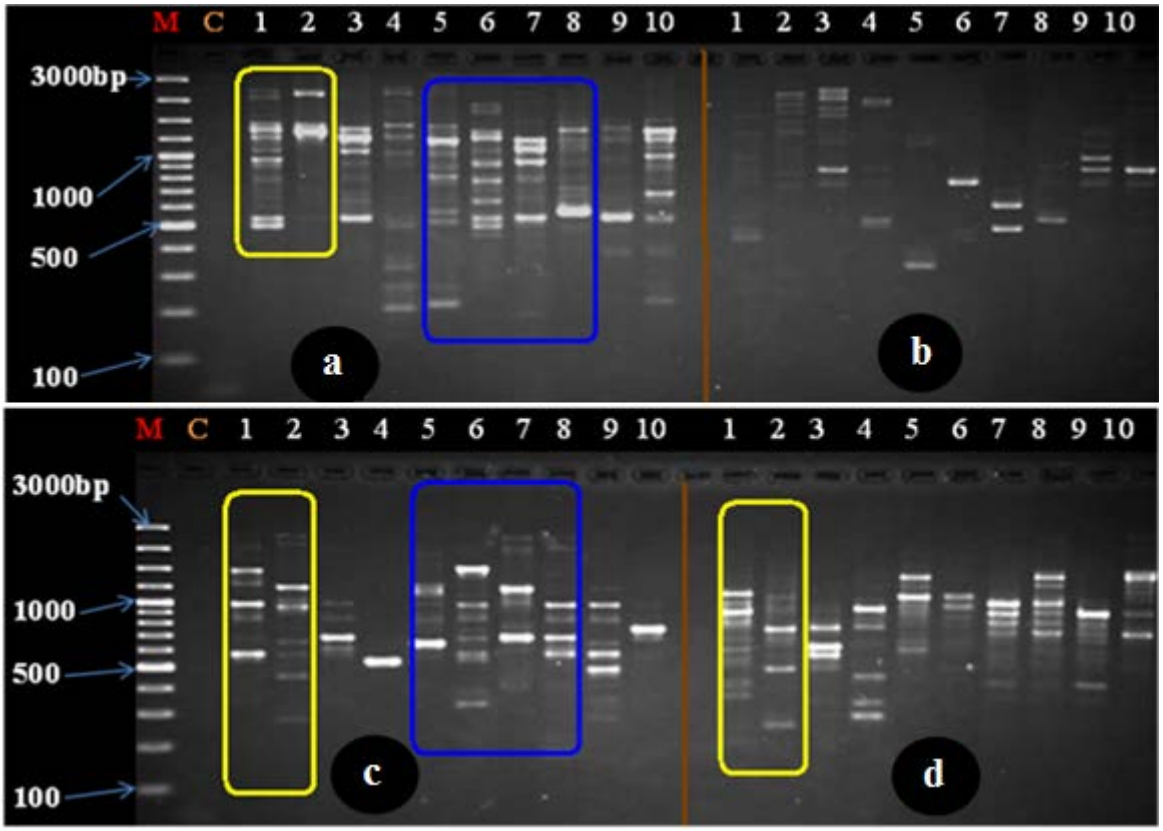

Figure 5.16: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPM-07, b: OPM-08, c: OPM-09 and d: OPM-10. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

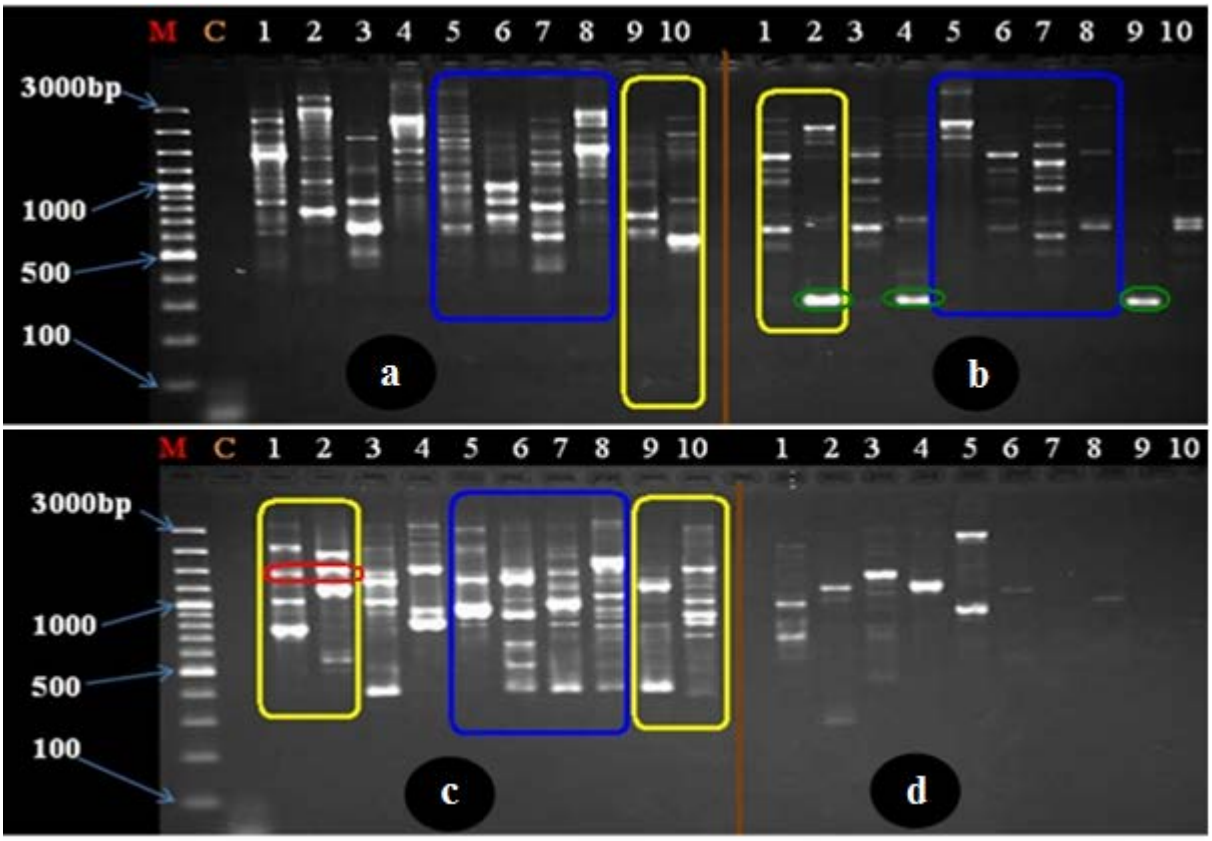

Figure 5.17: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPM-11, b: OPM-12, c: OPM-13and d: OPM-14. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

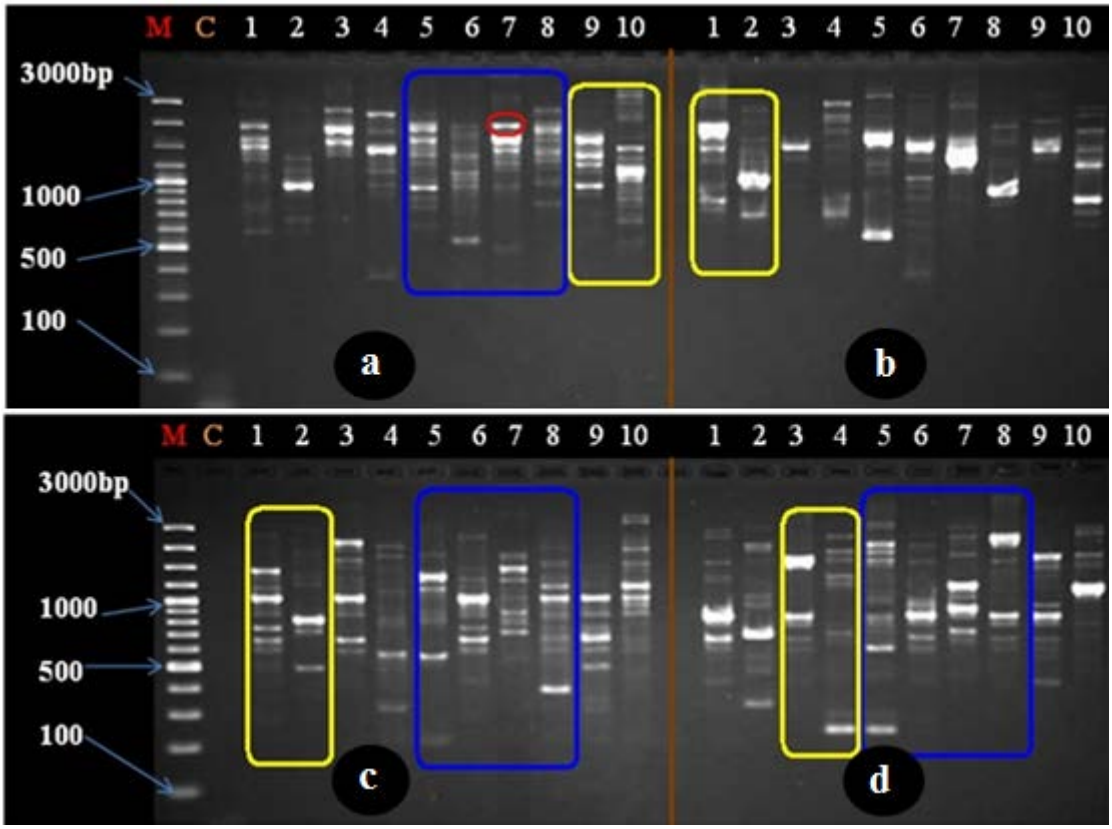

Figure 5.18: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: OPM-15, b: OPM-16, c: OPM-17and d: OPM-18. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

5.3.5 Microsatellites

Amplification was successful with all five microsatellite primers. The primers generated a total of 124 reproducible amplified products of ~180-3400 bp that showed polymorphism between and within the SSI DNA extracts (**Tables 5.5-5.6, Figs 5.19-5.20**). The profiles contained typically 3 to 14 bands and showed sufficient variation among the SSI extracts to be useful in genotyping. Primers HKB17/9 and $(GACA)_4$ showed the most polymorphism and HKB17/33 and $(GTG)_5$ the least.

Polymorphism in profiles was observed between successive imports of all SSI extracts, e.g. e1 with HKB17/9 (**Fig. 5.19a,** yellow box) and HKB-23/52 (**Fig. 5.19b,** yellow box), e6 with $(GACA)_4$ (**Fig. 5.20b,** blue box) and $(GTG)_5$ (**Fig. 5.20c,** yellow box).

Polymorphic profiles also differentiated among all four extracts of e3, both between original imports and among generations derived from them, *e.g*. with HKB-23/52 (**Fig. 5.19b**) and HKB-17/33 (**Fig. 5.20a,** blue box).

Some band sizes were common to several isolate extracts, e.g. at ~600 bp with HKB-17/9 (**Fig. 5.19a**, red circle). By contrast, monomorphic genotypes were shown by unique bands, e.g. with HKB-17/9 isolate 2 (e1b) had a unique band of ~1500bp and isolate 9 (e6a) had a unique band of ~130bp (**Fig. 5.19a**, green boxes).

Table 5.5: Microsatellite analysis results for single spore isolates.

Primers	**Total bands**	**Range of band sizes (bp)**	**Primers with most polymorphism**
HKB17/9	35	200 – 3200	HKB17/9 $(GACA)_4$
HKB17/33	18	180 – 3000	
HKB23/52	24	250 – 3000	
$(GACA)_4$	31	300 – 3400	
$(GTG)_5$	16	500 – 2800	
Total	**124**	**180-3400**	

Table 5.6: Summary of polymorphism detected using five microsatellite primers with *Plasmodiophora brassicae* single-spore isolates. **+++**, strong polymorphism, ++: moderate polymorphism, + weak polymorphism.

Primer name	**Polymorphism**
HKB17/9	+++
HKB17/33	+
HKB23/52	++
$(GACA)_4$	+++
$(GTG)_5$	+

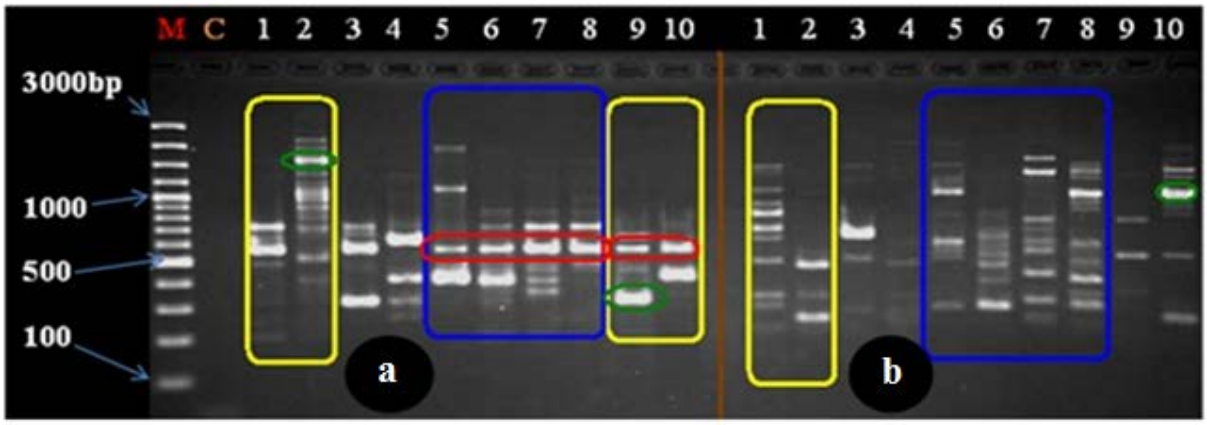

Figure 5.19: Microsatellite PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: HKB-17/9 and b: HKB-23/52. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

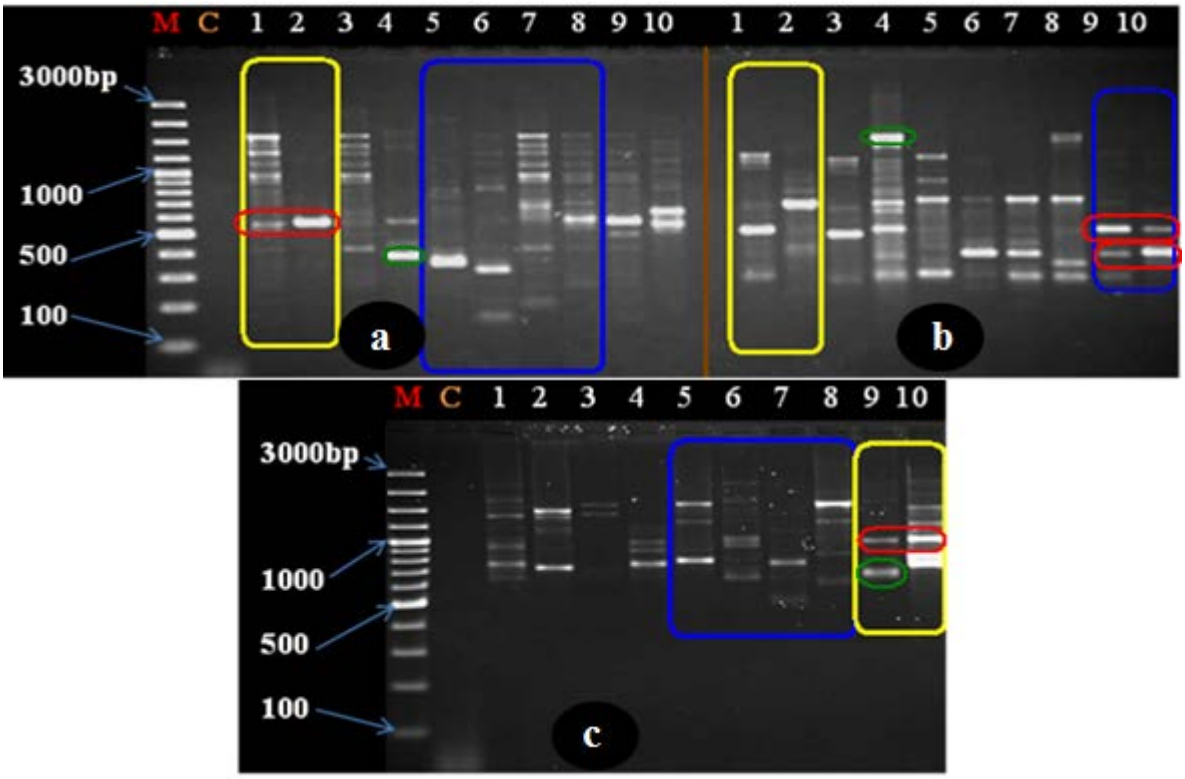

Figure 5.20: Microsatellite PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*. a: HKB-17/33, b: $(GACA)_4$ and c: $(GTG)_5$.Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate.

5.3.6 RAPD and microsatellite primers with SSI, plant and fungal extracts

The ten selected primers yielded a total of 459 amplicons (363 from the eight RAPD primers and 96 from the two microsatellite primers) (**Table 5.7, Figs 5.21-5.23**) with the 16 DNA extracts (from ten SSIs, four host plants and two fungi). Each primer produced 31-59 amplicons with sizes ranging from ~200 to 3500 bp.

All samples had different banding patterns (**Figs 5.21-5.23**). In particular, the profiles for the SSIs did not match those for the plant hosts or the contaminating fungi from the galls, e.g. with OPA-16 (**Fig. 5.21c**) and $(GTG)_5$ (**Fig. 5.23b**). The soil fungi used were isolated from galls of some SSIs and so were included in the analysis to check that differences in bands were not to their DNA.

Table 5.7: PCR analysis of 16 DNA samples (from 10 SSIs, 4 plants and 2 fungi) using 8 RAPDs and 2 microsatellite primers.

Primers	**Ten single-spore isolates of *Plasmodiophora brassicae***		**Four host plants**		**Two fungi**	
	Total bands	**Range of band sizes (bp)**	**Total bands**	**Range of band sizes (bp)**	**Total bands**	**Range of band sizes (bp)**
OPA-02	51	300-3000 bp	26	200-3500 bp	14	280-3000 pb
OPA-06	34	200- 2800 bp	16	400-3500 bp	13	500-3000 bp
OPA-15	44	500-3500 bp	15	600-3000 bp	6	700-2800 bp
OPA-16	37	600-3500 bp	12	400-2800 bp	10	500-3000 bp
OPB-04	55	300-3000 bp	13	300-2800 bp	12	300-3500 bp
OPB-11	52	200-3500 bp	26	280-3500 bp	-	-
OPB-14	31	280-3500 bp	13	500-3400 bp	9	200-3000 bp
OPM-07	59	200-3500 bp	29	220-3500 bp	9	220-2800 bp
Total bands	**363**		**150**		**73**	
$(GACA)_4$	54	300-3000 bp	28	200-3000 bp	17	300-3000 bp
$(GTG)_5$	42	400-3000 bp	12	500-2800 bp	8	500-2500 bp
Total bands	**96**		**40**		**25**	

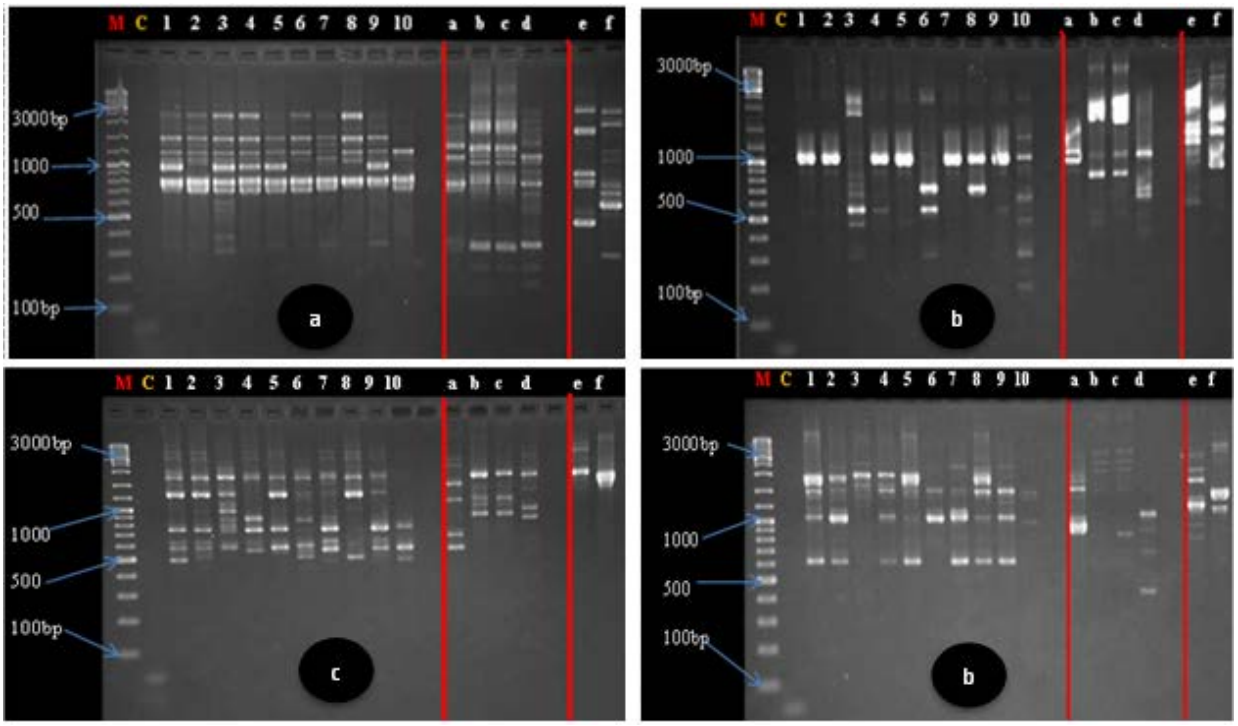

Figure 5.21: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*, 4 host plants and 2 contaminant fungi. a: OPA-02, b: OPA-06, c: OPA-15 and d: OPA-16. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate, a- broccoli, b- Chinese cabbage, c-cabbage, d- cauliflower, e- *Exophiala dermatitidis*, f- *Penicillium chrysogenum.*

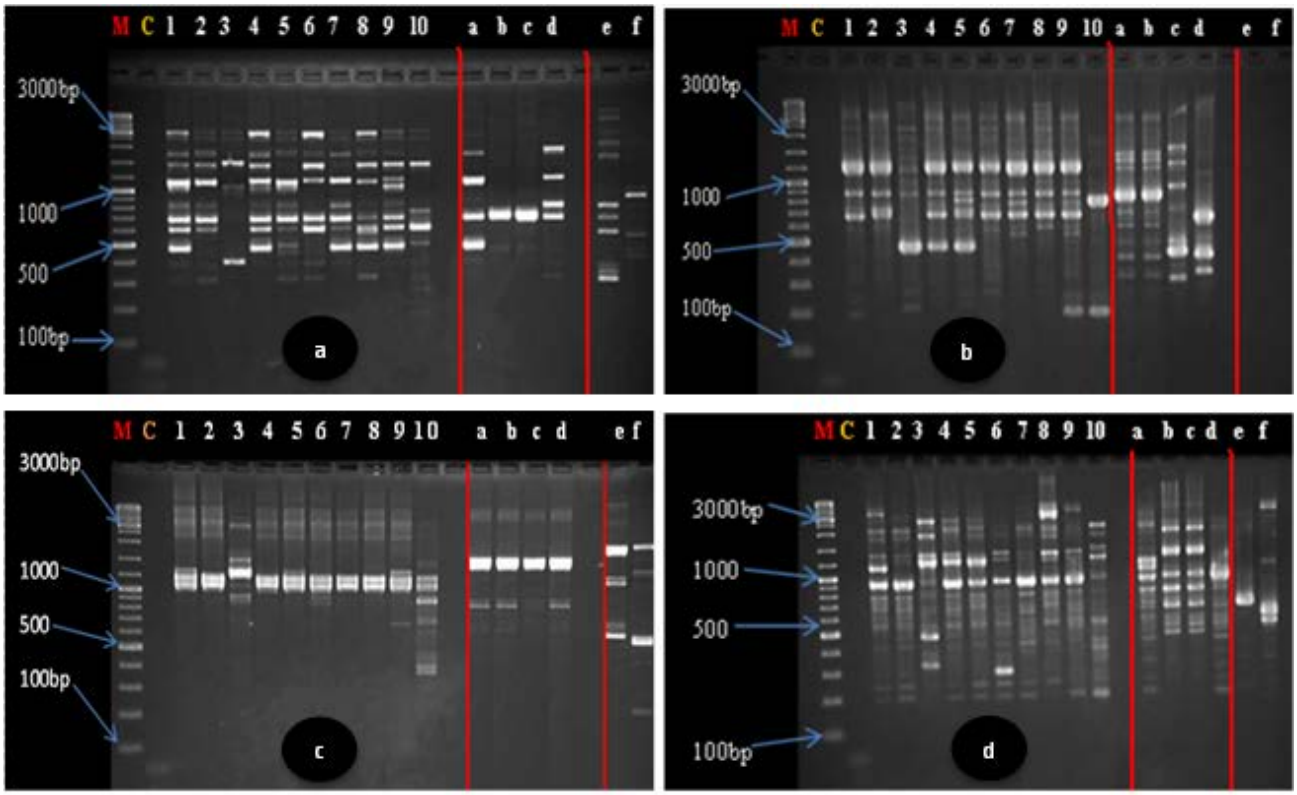

Figure 5.22: RAPD PCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*, 4 host plants and 2 contaminant fungi. a: OPB-04, b: OPB-11, c:OPB-14 and d: OPM-07. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate, a- broccoli, b- Chinese cabbage, c- cabbage, d- cauliflower, e- *Exophiala dermatitidis*, f- *Penicillium chrysogenum.*

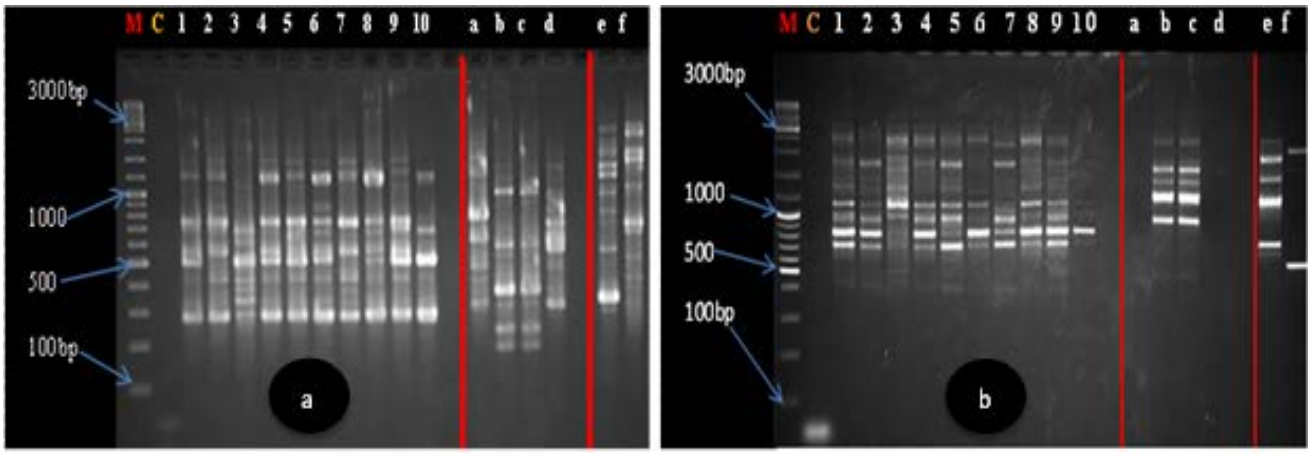

Figure 5.23: MicrosatellitePCR products from DNA of 10 single-spore isolates of *Plasmodiophora brassicae*, 4 host plants and 2 contaminant fungi. a: $(GACA)_4$ and b: $(GTG)_5$. Lanes are (left to right): lane M molecular weight marker (GeneRuler), lane C negative control; numbers from 1-10 refer to different single spore isolates: 1- e1 old single spore isolate, 2- e1 new single spore isolate, 3- e2 old single spore isolate, 4- e2 new single spore isolate, 5- e3 old single spore isolate. 6- e3 old single spore isolate generated in Victoria, Australia, 7- e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3 new single spore isolate, 9- e6 old single spore isolate, 10- e6 new single spore isolate, a- broccoli, b- Chinese cabbage, c- cabbage, d- cauliflower, e- *Exophiala dermatitidis*, f- *Penicillium chrysogenum.*

5.3.7 Multivariate analysis of single-spore isolates and generations

Both types of multivariate analysis showed that successive generations were not genetically identical (**Figs 5.24-5.25**). Some SSI generations were clustered relatively close together, e.g. e3 and e6, whereas others shared less than 10% similarity, e.g. e1 and e2. Both types of analysis showed the SSIs to be very different from the host plants and fungi.

Hierarchical cluster analysis showed that each successive generation had a maximum of 50% similarity to the previous one (**Fig. 5.24**). Successive accessions of SSI e6 were clustered in one clade at ~50% similarity but SSI e3 generations raised at DPI Knoxfield (D3) were only ~40% similar and only 20% similar to their original SSI e3a. For SSIs e1 and e2, successive accessions belonged in different clades and were less than 10% similar to one another.

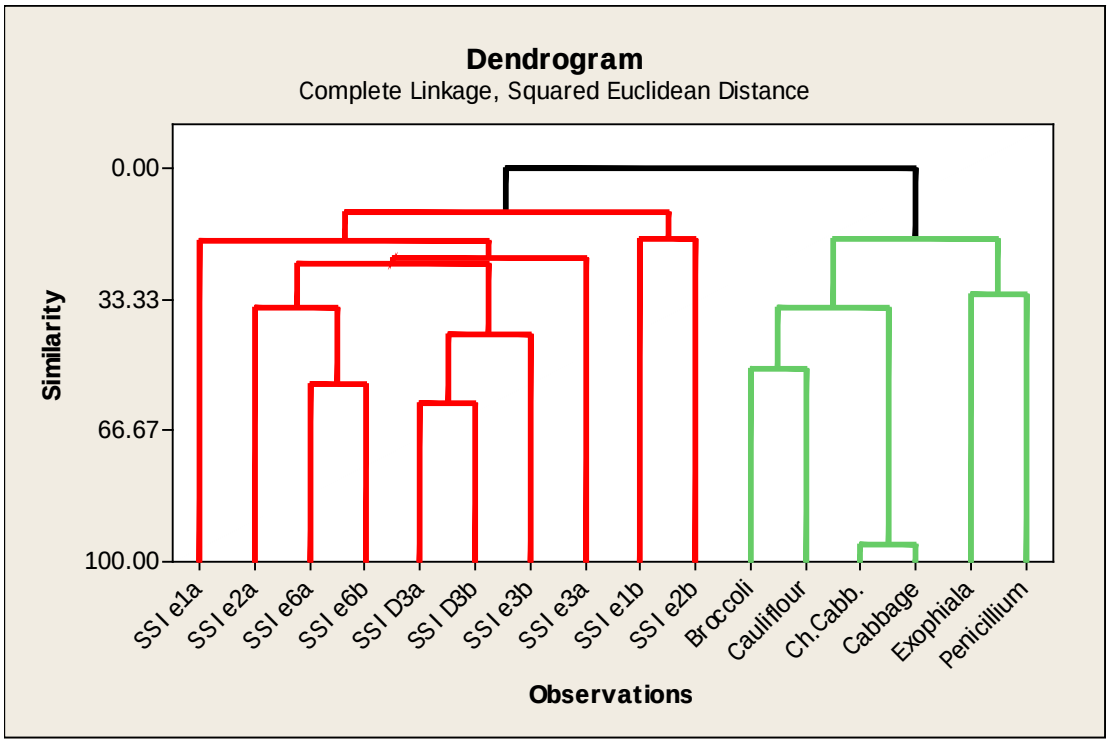

Fig. 5.24: Hierarchical cluster analysis of RAPD and microsatellite polymorphism in successive generations of single-spore isolates using complete linkage and squared Euclidean distance at p=0.05. Key: 1- e1a old single spore isolate, 2- e1b new single spore isolate, 3- e2a old single spore isolate, 4- e2b new single spore isolate, 5- e3a old single spore isolate. 6- D3a - e3 old single spore isolate generated in Victoria, Australia, 7- D3b - e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3b new single spore isolate, 9- e6a old single spore isolate, 10- e6b new single spore isolate.

Principal components analysis showed the same pattern as for hierarchical cluster analysis (**Fig. 5.25**). SSI e6 accessions were clustered together, as were e3 generations raised at DPI Knoxfield (D3), but old and new SSI isolates e1, e2 and e3 were widely scattered.

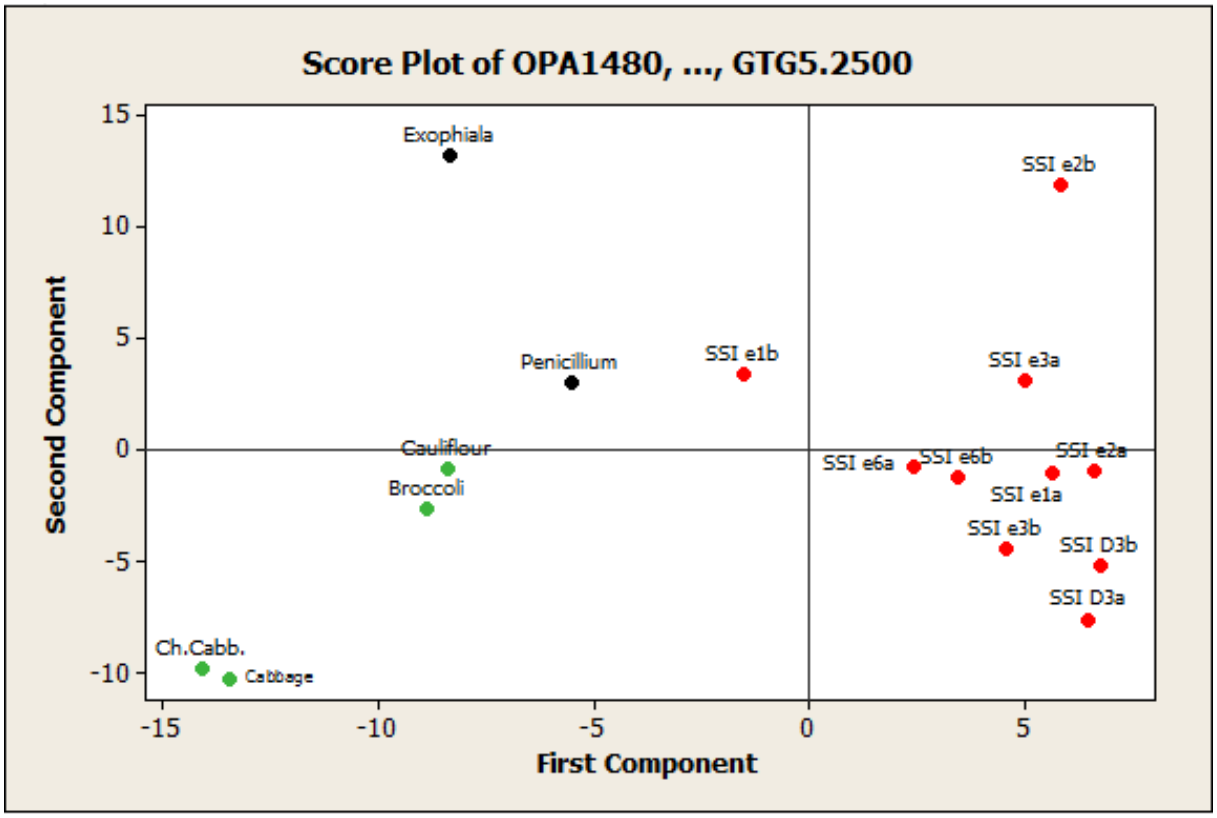

Fig. 5.25: Principal components analysis of RAPD and microsatellite polymorphism in successive generations of single-spore isolates at p=0.05.Key: 1- e1a old single spore isolate, 2- e1b new single spore isolate, 3- e2a old single spore isolate, 4- e2b new single spore isolate, 5- e3a old single spore isolate. 6- D3a - e3 old single spore isolate generated in Victoria, Australia, 7- D3b - e3 new single spore isolate generated from number 6 in Victoria, Australia, 8- e3b new single spore isolate, 9- e6a old single spore isolate, 10- e6b new single spore isolate.

5.4 Discussion

This is the first study conducted in Australia on the genetic changes that occur in different accessions and the first study to show genetic heterogeneity in PCR profiles of successive accessions and generations of single-spore isolates of *P. brassicae*. This is significant because it differs from the theoretical expectations from a SSI. If a single resting spore can generate a genetically diverse population in galls on the first plant it infects, the effort needed to generate SSIs may not be warranted, as they are unlikely to produce the homogeneity of results predicted. Breeding cultivars for resistance by selecting invariant target genes would inevitably be exceedingly difficult with a continuously genetically diverse population.

The polymorphism among SSIs e1, e2, e3 and e6 was expected, as noted by Klewer et al. (2001), who showed that e3 and e6 were more similar genetically to one another than to e1 or e2, as shown in the multivariate analyses in this study. Many other studies have found high DNA polymorphism in other SSIs generated from even single galls (e.g. Manzanares-Dauleux et al. 2001), but none of these studies considered the possibility of genetic variation through generations. SSIs are commonly bulked up for later experimentation and the differences noted here following that process for e3 could explain the differences between accessions. Any heterogeneity within *P. brassicae* in the galls could be selected out during this process (Jones et al. 1982a; Fahling et al. 2003). Klewer et al. (2001) found e3 and e6 to be invariant using OPA-10, unlike this study, but the process of bulking up SSIs through plant passage may have resulted in genetic variation in the intervening 12 years.

The high DNA polymorphism noted here differs markedly from the stability reported by Heo et al. (2009), in South Korea, who showed that SSIs e4 and e9 of *P. brassicae* were stable genetically in the next and the following generations, but used only two-three primers: URP 3 and 6 (20-mers) and OPA-07 (10-mer). Primers of 20 bases are less likely to discriminate than those of 10 bases and studying DNA polymorphism using small primers requires a large number to be used for adequate discrimination. The 60 RAPD and six microsatellite primers used in this study varied in discrimination among samples and injudicious use of only a few would miss the polymorphism. For example, Moller and Harling (1996) screened 40 RAPD primers against DNA from three unrelated SSIs and preparations of host plant starch grains; of these, 23 gave 1-6 bands per primer, five gave identical results for all SSIs and only three generated specific profiles for each SSI. Using only three primers with small numbers of

samples (Heo et al. 2009) reduced the chances of finding polymorphisms, especially with highly related SSIs.

Several explanations for the large amount of variation in the SSIs in this study may be suggested (lack of homogeneity in SSIs used, random mutation and artefacts of the techniques used) and these are discussed further below. Technical issues are discussed first, to evaluate the possibility of the variation being due to experimental artefacts. This is followed by discussion of the homogeneity of the single-spore isolates with respect to their origin and the *P. brassicae* life cycle.

5.4.1. Artefacts of the experimental process

5.4.1.1 Extraction of DNA

Working with tissue infected with the pathogen is very difficult, in terms of obtaining pure DNA of the pathogen (Faggian et al. 1999; Moller and Harling 1996). There are many contaminants that could hamper attempts to develop any molecular biology-based test for studying *P. brassicae*, in particular using non-specific RAPD and microsatellite primers. The resting spores are difficult to purify from their host plant tissue (galls) and are often contaminated with bacteria and other organisms (Bryngelsson et al. 1988; Faggian 2002). The utmost precautions were taken to prevent these factors affecting the results, although the possibility that the pathogen had taken up plant DNA could not be eliminated (see 5.4.2.4 later), and so the DNA of the various host plants was included as a target with the reduced set of RAPD and microsatellite primers that formed the final analysis.

DNA was successfully extracted from its sources (galls and resting spores) with sufficient purity for reliable PCR amplification of the target DNA. This does not, however, necessarily mean that the only DNA in the extracts was that of *P. brassicae*. Klewer et al. (2001) refer to the DNA yield from young galls inoculated with the ‘e’ series SSIs as being a mixture of ‘approximately equal amounts of host and pathogen DNA’ but it is unlikely that the proportion of DNA was as great as this in these extracts, due to the more exacting method of preparation, which should have removed any host DNA outside the resting spore wall, and extraction from spore suspensions rather than young galls.

It is unlikely that the differences noted were due to different levels of contamination of the DNA by host cell DNA during extraction from the galls or resting spore suspensions. The

extraction technique used followed that of Manzanares-Dauleux et al. (2001) when they examined the genetic diversity among nine single-gall populations of *P. brassicae* and 37 single-spore isolates (SSI); this involved peeling the galls and centrifuging the resting spores to exclude contamination of the SSI DNA with DNA of the host plants or any other organism, including microorganisms on and in the roots. The peeling process removes the outer layer of the root and cortex and is intended to reduce the proportion of host plant DNA as well as DNA from microorganisms. Also, treating the infected plant tissue with DNase, as recommended by Manzanares-Dauleux et al. (2001), would have reduced host plant and contaminating microbe DNA. It is unlikely that differences in the SSIs from gall tissue prepared by the same techniques was an artefact of DNA extraction, as the purity of the DNA from SSI spore suspensions should be greater than that from galls.

5.4.1.2 ITS and specific primers

PCR with the universal primers ITS1 and ITS4 showed only one band at ~700 bp, characteristic of *P. brassicae*, as found by Faggian (2000) and Kim et al.(2001), suggesting that there was no contamination from the host plant (~800-900 bp) or fungal DNA (~500 bp) that might have affected the non-specific RAPD and microsatellite-primed PCRs. As Faggian et al. (1999) found the ITS region to be essentially invariant, the bands were not sequenced, though it is possible (but unlikely) that the SSIs showed some differences. In retrospect, it might have been useful to sequence the bands to confirm this but the large degree of variation noted with non-specific primers made it redundant.

The specific primers PbITS1, PbITS2, PbITS6 and PbITS1 specifically amplified only the single bands expected from *P. brassicae* DNA but not the plant or fungal DNA and so for all ten SSI samples any changes in the isolates did not affect where the primers bound. Similarly, Klewer et al. (2001) found no differential amplification with the ‘e’ series SSIs used here.

5.4.1.3 Using RAPD and microsatellite primers to find genetic variation

Because PCR-based molecular analyses need only a small amount of biological material to work with their DNA, they are highly relevant to the investigation of obligate pathogens such as *P. brassicae* (Faggian and Strelkov 2009). There is, however, the danger with these non-specific primers of the amplicons not being from *P. brassicae* but DNA of the host plant or soil microbes. Despite this, both marker systems have been used successfully to characterize molecular variation in *P. brassicae* (including galls) by many researchers in many countries

(Buhariwalla et al. 1995; Moller and Harling 1996; Yano et al. 1997; Manzanares-Dauleux et al. 2001; Fahling et al. 2003; Osaki et al. 2008a; Rosa et al. 2010) by taking appropriate precautions.

The abundant reproducible polymorphism of the DNA from the SSIs and their progeny with both RAPD and microsatellite primers suggests that the variation observed was not an experimental artefact. The RAPD and microsatellite profiles for all samples showed clear polymorphism with 48/60 RAPD and all five microsatellite primers used, and the bands produced by DNA with the same primers from the plant hosts and possible fungal contaminants with the same primers were not the same as those produced by DNA from *P. brassicae*. The large amount of polymorphism in PCR products from the SSIs suggests the presence of genetic variation even in paired SSIs (original and subsequent accessions, or original accession and progeny), leading to their separation into multiple groups, some with little similarity to their original accessions.

The very large amount of variation in profiles of the 'e' SSIs and their progeny with both RAPD and microsatellite primers was surprising, e.g. e1 accessions, and e3 generations produced in Victoria, Australia. The 'e' SSIs accessions were genetically different from one to another and in both e3 generations their profiles were different. The diversity of the 'e' series SSIs was also examined by Klewer et al. (2001). Sequence-derived primers and RFLP produced reliable polymorphism in SSIs e1, e2, e3 and e6 as used here, but they found no differentiation using RAPD primers OPA-10, OPR-02 and OPR-12; by contrast, in this study OPA-10 produced distinctively different profiles with the same SSIs. SSIs e3 and e6 were genetically closer than to other SSIs in both the Klewer et al. (2001) study and here, and they differed in pathogenicity – e3 stimulated a resistant reaction in two out of four lines of *Arabidopsis thaliana,* whereas e6 did not (Klewer et al. 2001).

The unique PCR profiles for each of the SSI accessions and e3 progeny with 3/60 RAPD primers (OPA-10, OPB-12 and OPM-13) was comparable to the proportions of unique profiles for three unrelated SSIs shown by Moller and Harling (1996) and Manzanares-Dauleux et al. (2001) using some of the same RAPD primers.

The large differences in PCR profiles in three generations of SSI e3 were not expected. The only previous paper studying successive generations of SSIs showed that SSIs e4 and e9 retained the same DNA PCR profiles through three generations, but only three primers

(URP3, URP6 andOPA-07) were used in this South Korean study (Heo et al. 2009). Using a larger number of primers, as in the current study, would have increased the likelihood of finding differences, as discussed previously.

5.4.2 Genetic diversity in single-spore isolates

In theory, each SSI should have a unique molecular genotype, as the rationale for producing them was to eliminate the variation seen in field populations to facilitate breeding of clubroot resistance into host plants (Jones et al. 1982a). Yet, as found by others (Klewer et al. 2001; Manzanares-Dauleux et al. 2001), the SSIs had high DNA polymorphism. Explanations for this, particularly between original accessions and their progeny, are confounded by the uncertainties surrounding the life cycle of *P. brassicae* (as noted by Manzanares-Dauleux et al. 2001), but include: heterogeneity in the original accessions, sexual recombination, random mutation and incorporation of host DNA, which are considered below.

5.4.2.1 Lack of homogeneity in original accessions

One possible reason for the heterogeneity observed between successive SSI accessions is that the SSIs were not generated from a single uninucleate resting spore, but from at least one larger spore with 2-4 nuclei, as reported by Narisawa et al. (1996). The ‘e’ series SSIs (e1, e2, e3 and e6) were originally produced from the field isolate ‘e’ (Fuchs and Sacristan 1996) within ‘the institute’ (presumably the Institute of Genetics in the Free University of Berlin) by Schallehn *et al.* (unpublished) (Klewer et al. 2001; Fahling et al. 2003) but the method used was not detailed. The four ‘e’-derived SSIs varied in pathogenicity but e3 was most similar to the original field isolate ‘e’ by RFLP analysis (Klewer et al., 2001; Fahling et al., 2003). Scott (1985) selected larger resting spores as sources for SSI sources, yet according to Narisawa et al. (1996) these were more likely to be binucleate. If the ‘e’ series was similarly constituted, the original resting spores may have been heterogenous. Therefore it cannot be assumed that all resting spores are uninucleate and haploid. Alternatively, the original spores may have been homogeneous and random mutation through the various bulking up (plant passage) and storage (-20°C) may have resulted in heterogeneity that is magnified by sexual recombination. It would be interesting to study historical batches of bulked up materials to see if they also show DNA polymorphism. Without details of how the SSIs were made originally or genetic analysis of successive accessions through the intervening years, it is impossible to be certain.

5.4.2.2 Sexual recombination

The position of sexual recombination in the life cycle of *P. brassicae* is mostly commonly agreed as occurring during meiosis following karyogamy in the cortical plasmodia (Tommerup and Ingram 1971; Ingram and Tommerup 1972; Garber and Aist 1979b), but, as noted by Kageyama and Asano (2009), 'this hypothesis is not universally accepted'. Furthermore, large resting spores with 2 or 4 nuclei have been noted by Narisawa et al. (1996) and such large spores were selected by Scott (1985) in attempts to make SSIs.

Clubroot results from a primary infection of a host root by a single resting spore (**Fig. 1.3**-life cycle). The protoplast of this resting spore is released inside a host cell and cleaves into primary zoospores, which are then released, mate and return to the host root as secondary zoospores that form one or more galls. Early light microscope observations of meiosis in the initially binucleate plasmodium from secondary zoospores (Tommerup and Ingram 1971) have largely been confirmed by TEM studies (Garber and Aist 1979b). If the initial resting spore is dikaryotic and heterogeneous (from sexual recombination), the dikaryotic secondary zoospores that return to the host root and cause the galls, from which the next generation of resting spores of the 'e' accessions are formed, would give further genotypes through sexual recombination in the galls (Braselton 1982; Kobelt et al. 2000, cited by Siemens et al. 2009a). In this way, the next generation of resting spores is likely to be different genetically from the original spore, and moreover this process will continue to produce genetic diversity in the same manner. Further exploration of this would require isolation of both uni- and bi-nucleate resting spores and analysis of galls formed for DNA polymorphism.

5.4.2.3 Random mutation

The reason that the two SSIs e1 were located in different groups could be that the isolates were mutated by storage and transport conditions. On both occasions accessions were imported in this study, they were thawed out on arrival even though it was specified that accessions must remain frozen. This might have induced greater than normal mutation and so resulted in the large amount of variation seen with RAPD and microsatellite primers, by contrast with other studies (Heo et al. 2009).

Also, the process of routinely bulking up SSIs by repeated plant passage and long-term storage of galls at -20°C may have damaged the DNA and induced random mutations or

chromosomal rearrangements. Evidence for this is the frequent discovery that resting spore preparations from stored galls cannot infect *Brassica* seedlings (e.g. Kong Kaw Wa 2009).

5.4.2.4 Chromosomal rearrangement

An alternative explanation is that *P. brassicae* has the ability to rearrange chromosomes without sexual recombination and that this can explain the occurrence of variation among *P. brassicae* SSIs.

The chromosomal number in *P. brassicae* is controversial. Tommerup and Ingram (1971) concluded from light microscopy that n=5 for *P. brassicae*, but Garber and Aist (1979a) showed by transmission electron microscopy (TEM) that even serial sectioning during mitotic metaphase showed a 'reticulate mass of chromatin' 'not resolvable into discrete chromosomes'. Following this, Braselton (1982) suggested that 2n=20 from observation of 'synaptonemal complexes' in TEM of serial sections.

Electrophoretic karyotyping has suggested 13 chromosomal bands of 750-1900 kb from spheroplasts of resting spores (Ito et al. 1994), 6 bands of 680-1700 kb from plasmodia (Bryan et al. 1996) and 16 bands of 680-2200 kb giving a total genome size of 20.3 Mb from plasmodia derived from clubs of 'e' and e3 (Graf et al. 2001). The discrepancies are probably from differences in resolution of the techniques used and Graf et al. (2001) suggested that the most likely number is 2n=20 (Braselton 1982).

It is possible, however, that the chromosome number is not stable, despite Tommerup and Ingram (1971) claiming that it remained stable for 2 years in callus cultures of *B. oleracea*. If chromosomal rearrangement takes place, it could explain the differences between accessions and successive generations of SSIs. Although the targets on the DNA would not change, their relative positions would, and so the sizes and numbers of the amplicons with RAPD and microsatellite primers would change. Such a change would be unlikely to affect highly conserved regions of DNA, such as ribosomal DNA, but could occur in less highly conserved sections such as the ITS region, and might have been detected if it had been sequenced. It seems unlikely that such changes in the ITS region occur frequently, however, as the ITS region is almost identical in all *P. brassicae* sequenced so far. Complete sequencing of *P. brassicae* may help to resolve these questions.

5.4.2.5 Incorporation of host DNA

The large amount of apparent genetic polymorphism between original accessions and their progeny might be because of the uptake of host DNA by the pathogen during each infection cycle (Bryngelsson et al. 1988; Buhariwalla and Mithen 1995b; Buhariwalla et al. 1995a; Yano et al. 1997). Klewer et al. (2001) referred to the DNA from galls as being 'a mixture of approximately equal amounts of host and pathogen DNA', but it is unlikely that the proportion of host DNA was as great as that in the extracts in this study due to the method of preparation, which should have removed any host DNA outside the resting spores.

Bryngelsson et al. (1988) showed that the host plant DNA was incorporated into the resting spores as 'high molecular weight entities'. If that was the case, it is likely that it would contain intact ribosomal DNA that would react with universal primers ITS1/ITS4, but no amplicons at the size expected for plant DNA were seen.

Also, no amplicon in common with a host plant or a fungal contaminant was scored and analysed (as with the method of Manzanares-Dauleux et al. 2001). Whilst it does not exclude the possibility that the incorporated DNA may have been partially digested and so may not have shown amplicons, e.g. ~700 bp with OPB-14, that were characteristic of the host plants, it does seem unlikely that so many amplicons would be produced, especially when there were none with universal ITS primers. On balance, it seems unlikely that the large amount of polymorphism seen in these SSIs and their progeny was due to incorporation of host plant DNA. This could be tested by passaging the SSIs through different hosts, as done by Bryngelsson et al. (1988), and seeing if the DNA polymorphism changes according to the host.

5.4.3 Implications of genetic polymorphism in SSIs for plant breeding

The cluster analysis did not result in any major groups of isolates with high similarity coefficients, suggesting that all the isolates were genetically distinct even though it was expected that successive accessions or accessions and their progeny would be genetically identical or almost so. Such pathogen populations are more likely to overcome genetic resistance and the variation observed might explain the frequent loss or erosion of resistance to clubroot among *Brassica* genotypes (LeBoldus et al. 2012).

Extensive genetic variation emerging in each generation of *P. brassicae* must be an important consideration in choosing breeding strategies to attempt to develop durable clubroot resistance in *Brassica* species. These findings undermine the importance of using single-spore isolates instead of field isolates for both genetic studies of resistance and virulence surveys of the pathogen (Manzanares-Dauleux et al. 2001).

This instability of the pathogen even in SSIs suggests that there is continuous genetic change in the pathogen from one generation to another, which leads to the negation of any attempts to establish a program of breeding for resistance. LeBoldus et al. (2012) showed that variation in some single-spore isolates and populations of *P. brassicae* had the ability to erode resistance in some genotypes of canola (45H29 and 08N823R); this is a warning of the risk of the gradual collapse and the disappearance of any resistance bred into crops. Potential applications of the results reported in this study are the investigation of the instability of *P. brassicae*, enabling advice to be given to growers on choice of crop or cultivar, and monitoring genetic changes of the pathogen genotype after passage through different host plants between successive generations.

Since the key to understanding the rapid changes to genotype of *P. brassicae* occur in infected roots, Chapter 6 investigated morphological events occurring therein.

Chapter 6. The infection and development of differentially virulent *Plasmodiophora brassicae* in resistant and susceptible cultivars in Australia

6.1 Introduction

In Chapter 2, ten Australian populations of *P. brassicae* were assayed on the ECD set for pathogenicity and virulence (as assessed by the amount of galling on the root). The ten populations from Victoria and Western Australia resulted in ten ECD profiles. This is similar to the large diversity reported previously in Australia (Donald et al. 2006a; Kong Kaw Wa 2009) but an extraordinarily high diversity, compared with the dominance of a few ECD types in Canada (Strelkov et al. 2006; Strelkov et al. 2007), USA (Dobson et al. 1983), Europe (Toxopeus et al. 1986) and the Philippines (Bernard et al. 2006), and the reasons for it are not clear. The range of virulence, from low, e.g. population No. 1 from Boisdale, Victoria, to high, e.g. population No. 4 from Rosebud, Victoria, was also high, and resulted in a range of macroscopic symptoms, from mild to heavy galling of the roots.

Despite many attempts to understand the life cycle of *P. brassicae* and the stages in host plant infection, there are still many uncertainties and, over a century from after the original explanation of *P. brassicae*, its life cycle and pathology 'continue to be incompletely understood' (Donald et al. 2008). As reviewed by Donald and Porter (2009) and Kageyama and Asano (2009), *P. brassicae* has three common stages during its life cycle. The first is survival in the soil as resting spores, occasionally for up to 20 years. The other two, vital, phases are a primary phase occurring in the root hairs and a secondary cortical phase. Though these phases are well known, how they are affected by resistance in the plant host or virulence in the pathogen has been studied only infrequently, especially in Australia. Knowing how these affect the infection process could provide valuable information leading to the development of new resistant cultivars and control methods.

Recently, Rennie et al. (2013) showed that the proliferation of *P. brassicae* is contained within spheroid galls, and suggested that the formation of these structures are not indicative of complete resistance to clubroot. They proposed that the lack of pathogen spread in the spheroid gall tissues could result from an inhibition or a restriction of the myxameoboid phase of *P. brassicae*.

The aim of this chapter was to study morphological differences in the invasion of hosts of different resistance by pathogens of different virulence using Australian cultivars and strains. The following material therefore outlines known events during clubroot development.

6.1.1 Root structure

Roots are the targets (infection courts) for *P. brassicae* (Agrios 2005) and so their structure is reviewed briefly here. In clubroot, the roots become enlarged and malformed and fail to function normally. As a result the plant is subject to wilting during periods of high transpiration. The plants do not thrive and yields are greatly reduced if the plants do not die.

The apex of the root is called the root cap (**Fig 6.1**); this thimble-shaped group of thick-walled cells at the root tip serves as a "hard hat" to push though soil and so protects the growing point. Cells of the root cap slough off as the root advances through the soil. The cells of the root cap are produced by the actively dividing cells of the root meristem just behind the root cap. The old root cap cells may also lubricate the tip as it grows. The cells formed in the meristem just behind the root cap lengthen rapidly and push the root tip into the soil with substantial force (Raven et al. 2005; Evert 2006) (**Fig. 6. 1**). Infection by *P. brassicae* has not been reported in this tissue.

Generally, there are three main parts of the roots behind the root cap. The first is called the zone of division or meristematic zone (apical and primary meristem) (**Fig. 6.1**). This consists of the root meristem that supports root elongation and is found at the root tip just behind the root cap, which protects the tender meristem tissues (Evert 2006; Whiting 2011). The second is the zone of elongation, in which the cells lengthen. These do not become infected. The third is the zone of differentiation or maturation, in which the primary tissues differentiate and mature.

These primary tissues are listed below, from the external surface to the centre (Raven et al. 1992; Evert 2006), with brief comments on how *P. brassicae* affects them. As each is discussed in more detail later on, the reader is referred to Agrios (2005) and comprehensive references are not cited.

- Root hairs act as absorptive unicellular extensions of epidermal cells. These function as the major sites of water and mineral uptake. Root hairs are extremely delicate and subject to desiccation. In *P. brassicae*, they are the infection court for invasion by primary zoospores from the resting spores in the soil.

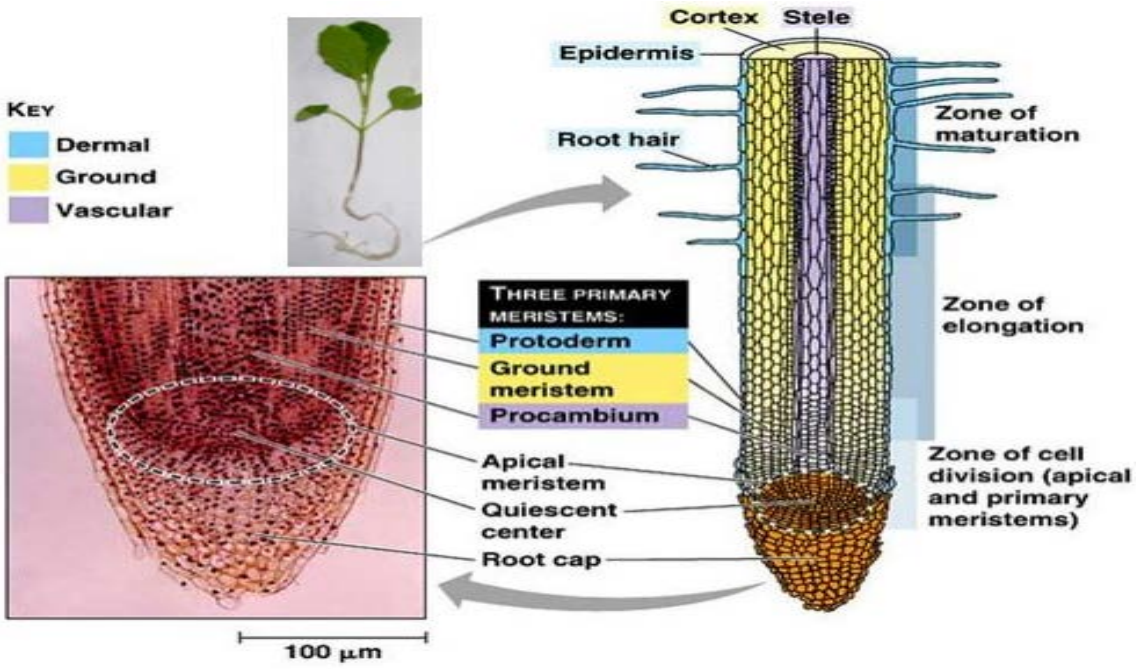

Figure 6.1: Root structure (http://www.baileybio.com/plogger/?level=picture&id=641).

- The cortex comprises several layers of parenchyma cells bounded on the outside by the epidermis and on the inside by the endodermis. *P. brassicae*, they are the tissue with most infection and contain three types of cell: infected cells occupied by amoeboid plasmodia arising from secondary zoospores, infected cells occupied by resting spores, and uninfected cells. The infected cortical cells show hypertrophy and the tissue shows hyperplasia, leading to disruption of the vascular tissues.
- The endodermis is a single layer of cells that separates the cortical tissues from the stele. Most cells have anticlinal walls containing suberin and so form a barrier to the entry of water and ions by the apoplast route. In *P. brassicae*, this seems not to act as a barrier to infection and there is no difference in the thickness of the walls with and without infection.
- Within the endodermis is the stele, which comprises the pericycle and the vascular tissue in bundles separated by parenchyma. The stele becomes infected and disrupted in *P. brassicae* infection.
    - The outermost layer of the stele is the pericycle, from which branch roots arise. *P. brassicae* infection of this layer may be responsible for hyperplasia.
        - The vascular system comprises bundles of xylem and phloem tissues with complementary functions: the phloem conducts materials and minerals

throughout the plant, while the xylem conducts water and minerals upwards from the roots. Anatomically, galls typically have vascular strands separated by large, proliferating parenchyma cells originating from secondary tissues. The xylem and the parenchyma become invaded and the secondary thickened walls of the xylem degraded.

6.1.2 Resting spore germination

The resting spore stage (**Fig. 6.2**) is one of the most important for infection and disease development, due to its ability to survive in soil for over 5 years despite the absence of its host plant (Gibbs 1931). According to Wallenhammar (1996), the half-life of viability in resting spores was 3–6 years and infection dropped to less than detectable in 17.3 years. Mature resting spores can be differentiated by scanning electron microscopy (SEM) (Buczacki and Cadd 1976; Ikegami et al. 1982; Williams and McNabola 1967). Mature spores were spherical to subspherical, about 3 µm diameter, with spines that covered the surface, while immature spores were surrounded with fibrous substances (Kageyama and Asano 2009).

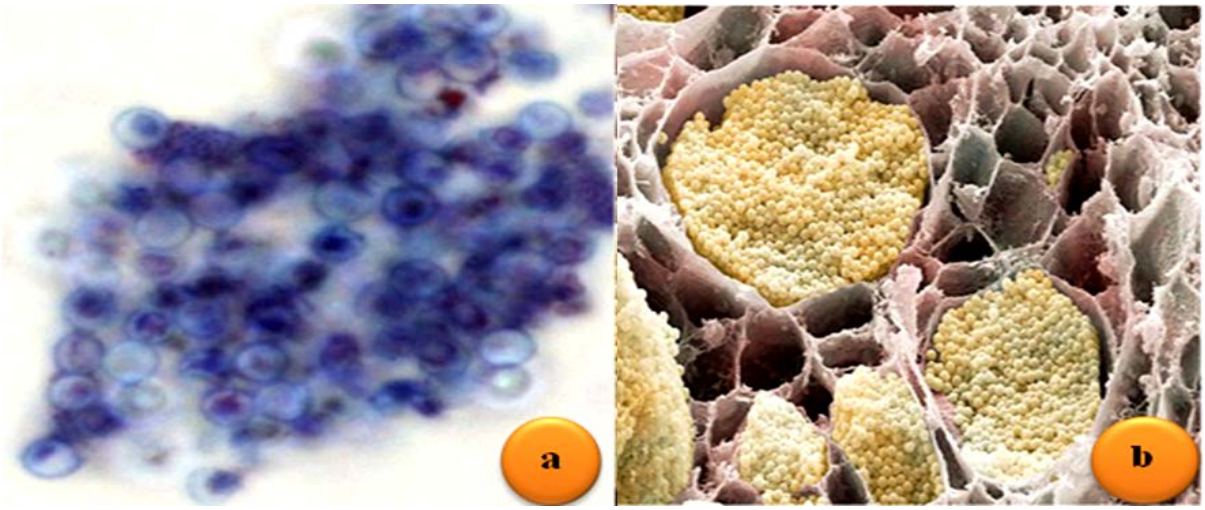

Figure 6.2: (a) Resting spores of *Plasmodiophora brassicae*, (a) Light micrograph of suspension of resting spores (b) Scanning electron micrograph (SEM) of gall cross-section showing the resting spores (yellow spheres). Source: Cabbage club root infection (*P. brassicae*) Science Image: http://www.psmicrographs.co.uk/cabbage-club-root-infection--plasmodiophora-brassicae-/science-image/18603d)

Germination of resting spores is the vital first stage in infection. Each viable resting spore germinates to produce a single zoospore, which encysts on and penetrates into a root hair in a host plant and develops there into a primary plasmodium that releases secondary zoospores. Mature resting spores from rotten galls had greater germination than those from younger hard galls (Macfarlane 1970; Yano et al 1991) and only reached 7-12% in laboratory conditions (Asano et al. 1999). The various environmental factors that affect resting spore germination have been reviewed by Dixon (2009a) and Donald and Porter (2009).

A critical factor in clubroot is that root exudates from host plants increase resting spore germination. The root exudates include inorganic ions, amino acids, amides, sugars, aliphatic acids, aromatic acids, volatile aromatic compounds, gases (such as ethylene), vitamins, peptides, proteins, enzymes, plant hormones, alcohols, ketones, olefins, urea and phytoalexins (Dundek et al. 2011). Wadhwa and Narula (2012) detected amino acids (arginine, threonine and lysine), sugars, and organic acids (citric, malonic, oxalic and succinic) in the root exudates of mustard (*Brassica juncea*) under laboratory conditions.

Macfarlane (1970) found that the germination of *P. brassicae* resting spores was increased by a diffusate from cabbage roots and so subsequent attempts to isolate SSIs used root exudates to increase resting spore germination (Jones et al. 1982a; Scott 1985). Yet this effect is non-specific, as Friberg et al. (2005) showed that aqueous root exudate preparations from the roots of perennial ryegrass stimulated the germination of resting spores under laboratory conditions. Also, Rashid et al. (2013) showed that root exudates of both host and non-host plant species stimulated resting spore germination. Furthermore, primary zoospores could infect non-host plants and produce primary plasmodia (Webb 1949; Macfarlane 1952) though the fate of the secondary zoospores is unknown and no infection of the cortex was reported.

In non-host plants growing under field conditions, resting spore germination stimulated by root exudates may reduce soil inoculum loads due to death of the zoospores in the absence of an appropriate living host (Kroll et al. 1984; Ikegami, 1985; Murakami et al. 2002; Friberg et al. 2005 & 2006) and therefore reduce disease severity in the following crops. The effect may, however, be non-specific and unimportant in the field, as Ahmed et al. (2011) showed that resting spore populations declined only marginally following two rounds of cruciferous crops (e.g. Chinese cabbage and canola) relative to non-cruciferous host crops (e.g. orchardgrass, perennial ryegrass or red clover) and non-host crops (barley and wheat).

6.1.3 The development of infection

Many researchers have attempted to understand the infection cycle of *P. brassicae* (Deora et al. 2012 and 2013; Donald et al. 2008; Feng et al. 2013; Gossen et al. 2013; Hwang et al. 2012a& b& c; Kobelt et al. 2000; Suwabe et al. 2003). As this necessarily involves roots, observations are hampered by soil and improved by hydroponic culture. Caution is needed in interpreting the various structures seen in fixed and sectioned material, as processing may introduce artefacts.

Dual cultures of callus and *P. brassicae* have been used to clarify the process (e.g. Tommerup and Ingram 1971) but suffer from cell pleiomorphy and the disorganisation of the tissues and so caution is needed in applying findings to whole roots. Hairy root cultures (Graveland et al. 1992; Asano et al. 1999; Asano and Kageyama. 2006) have been much more widely accepted and have even produced galls; in these, individual root hair infections can be followed chronologically and have largely confirmed events noted previously by others as well as providing new insights.

Some studies (e.g. Agarwal et al. 2008; Kobelt et al. 2000; Mithen and Magrath 1992) have used *Arabidopsis thaliana* as a model plant because of its small size, fast life cycle, small genome size and complete sequencing of its genome, enabling concurrent biochemical and genomic studies. Caution is desirable in extrapolating from events in *A. thaliana* to those in *Brassica* species, where galling is much more severe, though Mithen and Magrath (1992) and Kobelt et al. (2000) emphasised their similarities. Kroll et al. (1984) earlier pointed out inconsistencies between hosts in morphological and biochemical events and extolled caution in over-generalisation.

6.1.3.1 Primary phase

Generally, it is agreed that the resting spore in the soil germinates to produce a primary zoospore that infects a root hair, producing a primary plasmodium (Woronin 1878, cited in Kroll et al. 1984; Mithen and Magrath 1992). Asano et al. (1999) found that root hair infection occurred at 3-12 dai and estimated a time lag of 2 days before the release of primary zoospores from germinating resting spores. This is followed by the development of the primary plasmodium into zoosporangia (summarised by Kageyama and Asano 2009). When the primary plasmodium reaches a critical size, zoosporangia are formed inside the root hair and each releases 4-16 secondary zoospores that are capable of infecting further root hairs

and repeating the process (Naiki et al. 1984). These stages have also been observed in non-host plants (Macfarlane 1970).

Zoospores are commonly thought to be released externally because exit pores have been observed in the outside wall of the root hair (Ayers 1944) but have also been observed inside the lumens of root hairs (Aist and Williams 1971; Ingram and Tommerup 1972). Most of these secondary zoospores are uninucleate and identical in appearance to primary zoospores but about 10% are binucleate and a small proportion is quadri-nucleate (Narisawa et al. 1996). The origins of the multinucleate zoospores and what happens next are not clear. According to one view, some of the secondary zoospores fuse in pairs, with cytoplasmic fusion (plasmogamy) resulting in dikaryotic zoospores that initiate cortical cell infection (Tommerup and Ingram 1971; Ingram and Tommerup 1972). An alternative view is that secondary zoospores trapped in root hair lumens encyst within the root hair bases and release their protoplasts there (Aist and Williams 1971).

6.1.3.2 Secondary phase

Secondary zoospores are the key to cortical infection, as primary zoospores were stated not to infect cortical tissues (Dobson and et al. 1983). However, not all researchers agree that secondary zoospores are needed for cortical infection. Secondary zoospores may merely represent a short-term reinfection strategy (Buczacki 1983a). Cortical infections may be initiated by myxamoebae directly from primary plasmodia, as claimed by Mithen and Magrath (1992) on the basis of light and electron microscope studies of *A. thaliana*. These myxamoebae were described as 3-6 µm diameter, 1-2 nucleate, possessing pseudopodia and frequently being associated with breaks in the cell walls. While artefacts of processing cannot be excluded as explanations for these observations, Muhlenberg et al. (2002) showed by immunolabelling that they possessed cellulase. Mithen and Magrath (1992) pointed out that similar uninucleate myxamoebae had been observed earlier (Kunkle 1918; Dekhuijzen 1979; Ikegami et al. 1982) and Donald et al. (2008) also observed uni- or di-nuclear amoeboid structures by TEM in *B. oleracea* (cauliflower).

Infection of the cortex is the first step in the spread and diffusion of infection and disease within the host tissue (Donald et al. 2008) but how the initial infection of cortical cells occurs is still obscure. Small uni- and bi-nucleate plasmodia are observed in the outer cortex and are followed by secondary plasmodia that are initially binucleate but become multinucleate. In infected cortical tissue, plasmodia are found at different stages of development (Suwabe et al.

2003); young secondary plasmodia were located mainly in small cells next to the central cylinder whereas mature resting spore-forming plasmodia were located in large cells of the cortex near the periderm (Kobelt et al. 2000). Ingram and Tommerup (1972) suggested that the haploid nuclei in a multinucleate secondary plasmodium fuse in pairs to give a diploid nucleus, which immediately divides by meiosis, and each nucleus (with a fragment of cytoplasm) develops into a resting spore. By contrast, Mithen and Magrath (1992) proposed that karyogamy following plasmogamy of heterogeneous myxamoebae could result in multinucleate plasmodia, genetic recombination, increasing genetic and pathotype diversity and instability. However derived, multinuclear plasmodia differentiate into resting spores with chitin-protein walls and mature resting spores are observed simultaneously with all previous stages in sections through infected roots.

Characteristics of cortical invasion in susceptible plants are disturbance and breakage of the cell wall; vesicles or inclusion bodies inside the cell; increases in cell wall thickness and frequency of ‘cell wall stubs’; and elongation, multiplication and disorganization of the host nuclei (reviewed by Kageyama and Asano 2009). The disruption of cell walls is usually found in association with the amoeboid form of the pathogen, and it is presumed that this is the form of the pathogen that penetrates the cortical cells (Donald et al. 2008; Kobelt et al. 2000). In *A. thaliana*, Mithen and Magrath (1992) showed myxamoebae apparently protruding into adjacent cells through breaks in the cell wall. The myxamoebae were associated with cytoplasmic extrusions into the vacuoles, host plant nuclei and amyloplasts in the host plant cytoplasm (Mithen and Magrath 1992). Donald et al. (2008) showed that myxamoebae occurred similarly in *Brassica oleracea* (cauliflower).

Gall formation results from hypertrophy and hyperplasia in infected roots of the host as well as in suspension culture (Gustafsson and Flat 1986; Ludwig-Muller and Schuller 2008; Mithen and Magrath 1992; Williams 1966). Hypertrophy results in the swelling of infected cortical cells and hyperplasia in cell division; both contribute to the rupture of cell walls, even in the vascular tissue. In susceptible hosts, infection makes the cortex lose its integrity (Kobelt et al. 2000; (Kong Kaw Wa 2009)et al. 2008). Hypertrophy is possibly the result of increased auxin production in the infected cells (Ludwig-Muller 1999a) and does not occur in uninfected cells. Hyperplasia occurs in both infected and uninfected cells in cultured gall tissue and was thought to arise from the ability of secondary plasmodia to synthesise cytokinins (Muller and Hilgenberg 1986). In *A. thaliana*, Kobelt et al. (2000) suggested that hyperplasia arose if the pathogen infected meristematic tissue in the pericycle or cambium.

Macroscopically, hyperplasia results in the loss of the distinction of cortex and stele (Kroll et al. 1983; Mithen and Magrath 1992) and the emergence of macroscopic club-shaped galls on the roots; Mithen and Magrath (1992) noted extensive infection and hypertrophy of infected cells 10 dai before a rapid increase in cell number by cell division. The galls and their structural disorganisation hamper the movement of water and minerals within the plant, which affects plant growth and production (Dixon 2009a; Manzanares-Dauleux et al. 2006).

6.1.3.3 Effect of experimental conditions

As studies have used a variety of experimental conditions, timings and events are not directly comparable even within one plant and pathogen combination. This can cause problems in extrapolating from one study to another. Medium (especially calcium level), pH and temperature are common experimental conditions that vary among studies.

Donald and Porter (2004a) showed that the development of infection in the root hairs was affected by the level of calcium but that this effect depended on pH. Root hairs rapidly became infected at pH as low as 5.5 but increasing the level of calcium to 20 mM significantly postponed the developmental progress of the pathogen; however, at pH 6.5, all calcium concentrations significantly delayed development. As pH increased, the number of infected root hairs and the effect of calcium amendment reduced for all treatment, e.g. at pH 8, 75–95% of root hairs were uninfected 10 days after inoculation and calcium had no significant effect on development.

The timing and amount of root infection is also affected by temperature (**Table 6.1**) (Sharma et al. 2011a, b). Root hair infection was greatest and earliest at 25°C, intermediate at 20°C and 30°C, and least and latest at 15°C and 10°C. Moreover, the full range of disease symptoms developed only over 15°C. This must be considered in comparing studies of infection incubated at different temperatures.

Table 6.1: Number of days after inoculation (dai) until the first observation of each stage of infection in the life cycle of *Plasmodiophora brassicae* at different temperatures (Sharma et al. 2011a).

Life cycle stage of *P. brassicae*	Days after inoculation				
	10°C	**15°C**	**20°C**	**25°C**	**30°C**
Primary zoospores	6	4	2	2	2
Primary plasmodia	6	4	4	2	4
Young zoosporangia	-	10	6	4	6
Mature zoosporangia	-	14	6	4	6
Partially empty zoosporangia	-	16	8	6	8
Fully empty zoosporangia	-	18	8	6	8
Infection of epidermis (and cortex)	-	20	10	6	10
Swelling of tap root	-	28	14	10	14

6.1.4 Differences with plant resistance and pathogen virulence

6.1.4.1 Primary phase

Most reports suggest that the early phases of infection in the root hairs appear to be unaffected by resistance in the host plant. The degree of host resistance made no difference to the amount of root hair colonisation (Ayers 1957; Dekhuijzen 1979; Macfarlane 1955). Kroll et al. (1983) found no differences in the rate or amount of primary phase (root hair) infection in susceptible, partially resistant and resistant (no galls) radish. Similarly, Donald et al. (2008) found normal root hair stages in both susceptible and resistant cauliflower. By contrast, the proportion of root hairs infected was greater in susceptible (59%) than resistant (49-51%) canola (*B. napus*) (Deora et al. 2013). The maximum in primary infection for compatible interactions was 65-70% while, in intermediate interactions, infection was significantly less (59%).

The interaction between plant and pathogen types also affects outcomes. An interesting observation was that the intensity of root hair infection was not correlated with disease index in 16 cultivars of crucifers with two pathotypes of *P. brassicae* but that it varied between pathotypes (Naiki et al. 1984), suggesting that it is cortical cell infection that is important rather than the initial root hair infection. Similarly, Tanaka et al. (2006) observed that root hair colonisation of the resistant *B. rapa* ssp. *pekinensis* cv. Kukai 70 (containing CR genes

from European fodder turnip) was greater than that of the susceptible cultivar Nozaki Nigo with the Hagi population of *P. brassicae*, but that cortical cell infection was greater in the susceptible than in the resistant plants, and commented that the same occurred in other CR gene lines (Dekhuijzen 1979; Kroll et al. 1983; Yamagishi et al. 1986).

Kong Kaw Wa (2009) used arrays and qPCR to study early differences in RNA during infection in Chinese cabbage (*B. rapa* var. *chinensis* cv. Granaat) with different levels of resistance to one Australian strain (S) of *P. brassicae*. He showed by microarrays and qPCR that genes for chitinase and superoxide dismutase were up-regulated and those for lipase down-regulated in partially resistant 'Tahono' compared with the susceptible 'Granaat' at ≤2 days after inoculation (dai). This suggests that resistant plants recognised *P. brassicae* at the early root hair colonisation phase and that hypersensitivity was invoked, but concluded that resistance was mainly associated with constitutive expression of genes for pathogenesis-related proteins.

6.1.4.2 Secondary phase

Hypersensitivity was associated with resistance in *A. thaliana* (Kobelt et al. 2000) and some *Brassica* species [*B. campestris* (Dekhuijzen 1979) and *B. rapa* (Takahashi et al. 2006)]. It was not associated with resistance in *R. sativus* (Kroll et al. 1983) or other *Brassica* species [*B. napus, B. nigra, B. oleracea* and *B. rapa* (Morgner 1995, cited in Kobelt et al. 2000)] and *B. rapa* ssp. *pekinensis* (Tanaka et al. 2006). In *A. thaliana*, necrotic tissue typical of hypersensitivity was located microscopically by autofluorescence and surrounded infected cells, and yet infected tissue developed plasmodia, resting spores, hypertrophy and even hyperplasia inside the necrotic cells (Kobelt et al. 2000). Hyperplasia was thought to occur if the pathogen colonised meristematic tissue in the pericycle and cambium (Kobelt et al. 2000) and this disorganised the tissues of cortex and stele, leading to classification as susceptible.

A main difference between resistant and susceptible plants is the appearance of cell wall damage in the susceptible host plants during infection by the pathogen; this has been observed in *A. thaliana* (Mithen and Magrath 1992; Kobelt et al. 2000) and *Brassica* species (Donald et al. 2008). This ranges from highly frequent in *A. thaliana* to infrequent in several *Brassica* species (Dekhuijzen 1976a; Gustafsson et al. 1986; Morgner 1995, cited in Kobelt et al. 2000). A lower level of cell wall rupture in resistant plants may restrict the movement of the amoeboid form in the cortex (Donald et al. 2008; Takahashi et al. 2006). Also, Donald et al. (2008) observed all stages of root hair and cortical infection by transmission electron

microscopy, including cell wall breaks associated with amoeboid cells, in both resistant and susceptible cauliflower but found that only susceptible plants had cell wall damage in the inner cortex and xylem.

Resistance to *P. brassicae* is hard to define. In *Brassica* species, several cultivars do not form macroscopically visible galls but still show all stages of the pathogen life cycle. Partial resistance is also known and typically delays, but does not prevent or necessarily reduce, gall formation (e.g. Kroll et al. 1983; Kobelt et al. 2000; Kong Kaw Wa 2009). It seems, therefore, that these are not 'resistant' but rather 'tolerant' by the definition of Crute (1986), as pointed out by Donald et.al. (2008); resistance is quantitative rather than qualitative and resistance likely to be horizontal and polygenic (Van der Plank 1963, in Agrios 2005).

Resistance is associated with reduced incidence of infection of cells in the root. Morgner and Sacristán (1995) noted reduced frequency of pathogen structures in resistant cultivars of two *Brassica* species: *B. napus* and *B. oleracea*, in the latter of which infected cells were very infrequent. In *A. thaliana* ecotypes, the proportion of infected cells in sections through hypocotyl and root varied with resistance from 8% in *Ze-0* to 35% in *Tsu-0*, ecotypes of different levels of resistance, but no comparable figure was given for the susceptible ecotype *Cvi* (Kobelt et al. 2000). The proportion of infected cells in all ecotypes increased with inoculum dose and time. Tanaka et al. (2006) also noted that infected cells were less frequent in a resistant than a susceptible cultivar of *B. rapa* ssp. *pekinensis*, in that they had to search through more sections to find them in resistant than susceptible roots, but this was not quantified. Also, in secondary infection of canola, at 28 dai the proportion of the area infected varied from 18-29% and was greatest in susceptible plants. Pathogen population also affected secondary colonisation, which was greater (32%) for a population from a field where populations were classified as pathotype P3 in 2006 (Strelkov et al. 2006) than for other inocula (Deora et al. 2013).

Delay and reduction in the production of highly multinucleate plasmodia and resting spores also characterises resistance, but some resting spores are always present, even in small spheroid galls on resistant cultivars of *B. rapa* ssp. *pekinensis* (Osaki et al. 2008b). Kroll et al. (1983) found in radish that the total number per root cross-section of uni- and bi-nucleate plasmodia was greater in susceptible plants than in resistant plants at early stages of cortical infection. In susceptible plants, the proportion of young uni- or bi-nucleate plasmodia declined with time as multinucleate and then 'diploid' plasmodia, and finally resting spores,

developed. By contrast, in resistant plants the proportion of uni- and bi-nucleate plasmodia remained high and no further development took place. Partially resistant plants were intermediate and had 12-day-delayed development of multinucleate secondary plasmodia and resting spores as well as reduced numbers of all categories. In *A. thaliana*, spores were produced in the susceptible *Cvi* ecotype in 17 days, but not until 29 dai in the resistant ecotypes *Ta-0, Tsu-0* and *Ze-0* (Kobelt et al. 2000). In *B. rapa* ssp. *pekinensis*, resistance in 'Kukai' was associated with reduction in the proportion of plasmodia reaching 30 nuclei and with a lack of resting spore formation (Tanaka et al. 2006). These observations support the suggestion of a plasmatic defence by the host (Morgner and Sacristan 1995). A puzzling contradiction is that some authors (e.g. Morgner and Sacrisán 1995) have described the disappearance of cortical infection at later stages in resistant plants whereas others have not (e.g. Donald et al. 2008).

Starch is believed to be the carbon source for *P. brassicae* in host tissue (Keen and Williams 1969) and lack of it in infected cells may relate to the delays observed in the development of plasmodia and resting spores in resistant plants. Graveland et al. (1992) showed that susceptible plants produced more than ten times the amount of starch grains than resistant plants. Additionally, the kinds of starch built up differed; in susceptible plants, infected cells synthesised large quantities of amorphous amylopectin, while uninfected cells of resistant plants built up crystalline amylose. Starch accumulated in infected cells in susceptible plants of *Brassica* species (Keen and Williams 1969; Morgner 1995, cited in Kobelt et al. 2000; Tanaka et al. 2006) and *A. thaliana* (Evans and Scholes 1995), whereas it was absent in resistant plants. Ito et al. (2001) found by differential screening of randomly amplified cDNA that one fragment, H6-2, was down-regulated in resistant plants of Chinese cabbage and had high homology to *A. thaliana* chloroplast 16S rRNA gene; they proposed that it was related to down-regulation of starch synthesis in amyloplasts, thus denying plasmodia a carbon source. Donald et al. (2008), however, noted the presence of amyloplasts in infected cells of both susceptible and resistant cauliflower and the presence also of what were thought to be aberrant plastids. A related observation is that infected cells in *Brassica* plants increased up to ten times in ^{14}C labelling from feeding of ^{14}C-glucose (Stewart 2008).

Active resistance-related defence reactions observed include reactive oxygen species (ROS) accumulation (by diaminobenzidine staining) in the endodermis, pericycle and vascular cambial regions in resistant but not susceptible plants and lack of lignification in the xylem in susceptible plants of *B. napus* (Deoora et al. 2013). This is consistent with the up-regulation

in susceptible plants of genes for detoxification of ROS and down-regulation of genes for lignin biosynthesis (Cao et al. 2008). This also fits with the up-regulation of peroxidase and superoxide dismutase (SOD) in partially resistant *B. rapa* var. *chinense* (Kong Kaw Wa 2009). The timing of defence-related indole glucosinolate production also varied between susceptible and resistant hosts 2 weeks after infection (Ludwig-Müller et al. 1999c). Both types of host plant possessed similar levels, but in susceptible plants the level increased after 14 and 20 days, while in resistant plants the level increased later, at 14 and 30 days after infection. This difference was considered to be a defence response in the resistant plants and the timing suggests that it was initiated during nuclear multiplication in the secondary plasmodia.

6.1.5 Aims of the anatomical study

The main aim of this chapter was to determine the differences between the invasion events when two Australian *P. brassicae* pathotypes with different degrees of virulence colonised two types of cabbage (*B. oleracea*) plants (susceptible and resistant). Specifically, this study aimed to document the morphological effects of pathogen virulence in host plants that vary from resistant to susceptible. In this way, it was hoped to differentiate events controlled by the pathogen and host. **The research question was: what differences are observed morphologically with variation in virulence in the pathogen and resistance in the host?**

The plant species used in the full study was cabbage because a resistant variety that does not develop macroscopically visible galls was commercially available along with a susceptible variety. The clubroot pathotypes were chosen from the ECD study in Chapter 2 and varied in rating from high to low virulence. The infection process was examined from invasion to gall development to document differences in critical stages in a hydroponic system under controlled conditions. Events at macroscopic and microscopic levels were followed by collecting the infected roots at intervals and fixing, embedding and sectioning for both light and electron microscopy. The histological study of the galls between plant hosts varying in resistance was compared with that from partially resistant and highly susceptible Chinese cabbage (*B. rapa* var. *chinensis*) in which gene expression had been studied by microarray and qPCR techniques (Kong Kaw Wa 2009).

6.2 Materials and Methods

6.2.1 Pathogen materials

For cabbage (*Brassica oleracea*), galls collected from Chinese cabbage plants used in Chapter 2 (ECD) and stored at -20°C were used to prepare inocula. Two populations were selected based on their ECD assay: population No.1 of low virulence and population No.4 of high virulence.

For Chinese cabbage (*B. rapa* var. *chinensis*), PhD student Stephane Kong Kaw Wa had inoculated Chinese cabbage cv. Granaat in 2008 with the 'highly aggressive' *P. brassicae* population S (ECD code 16/0/00) (collected less than 3 years previously from Launching Place, Victoria) (Kong Kaw Wa 2009). Galls from this experiment were donated in 2008.

6.2.2 Plant host materials

For cabbage (*B. oleracea*), the target host plants were susceptible and resistant lines of cabbage from Syngenta; the susceptible line was Stock ID CA0826 and the resistant line (no visible gall development) was 'Maxfield F1 CRR'. Diederichsen et al. (2009) explain the origin of this and the resistant cauliflower line used by Donald et al. (2008), both of which have the European CR genes. Note that cabbage cv. Kilaton and cauliflower cv. Clapton were renamed for the Australian market as cabbage cv. Maxfield and cauliflower cv. Highfield (C. Donald, pers. comm.). Resistance is known to be polygenic in *B. oleracea* (Crute et al. 1980).

For Chinese cabbage (*B. rapa* var *chinensis*), the target host plants were the susceptible ECD05 cv. Granaat and a partially resistant F_1 hybrid line (H06 – 'Tahono' CR-1-1) kindly provided by the Henderson Seed Group Pty Ltd, Victoria (Kong Kaw Wa 2009). No complete resistance (no galls formed) is available in this species but resistance is known to be monogenic (Crute et al. 1980).

6.2.3 Hydroponic system

A hydroponic system (Kong Kaw Wa 2009) was used because of its significant advantages. This involved growing plants using a mineral nutrient solution; following the symptoms during infection stages was easier than in soil and reduced the difficulties that occur with soil, e.g. the growth of contaminating microorganisms that could affect disease development. It also had other advantages: uniform clubroot spore concentration, less space requirement than

pots, ease of tissue collection, and good growth conditions such as uniform temperature, high moisture levels and pH range optimised for infection and disease development. These systems are usually pathogen-free during the experiments, allowing successful inoculation, infection and symptom development (Matheny 1995; Kong Kaw Wa 2009).

6.2.3.1 Setup and preparation of the hydroponic system

Hydroponic tanks were constructed from 27.5 L black plastic tubs (Robusta®) (59.5 cm × 41.0 cm × 18.5 cm) by puncturing their lids with 32 35 mm-diameter holes (8 hole × 4 hole grid) (**Fig. 6.3**) Up to eight hydroponic tanks at any one time were aerated with an aquarium air pump (Aqua One™, Model SR 9500) that was set to a high air output of 9.5 L/min. Each hydroponic tank was filled up to 20 L with a modified Hoagland nutrient solution (**Table 6.2**) adjusted to pH 5.5. All tanks were placed in a controlled growth room at RMIT University with a temperature of 21±4°C, humidity of 70-90% and an 18 h photoperiod (250 W halogen bulbs about 2.3 m above the plants at an intensity of ~270 μmoles m^{-2} s^{-1}) **(Fig. 6.3).**

6.2.3.2 Preparation of host plants

6.2.3.2.1 Cabbage

Seeds were treated with 5 mL of 5 g/L of Mancozeb Plus® contact fungicide (Yates™, NSW) for 30 min (not longer to avoid absorption of the pesticide) and surface-sterilised for 10 min in NaOCl (2% active chlorine), followed by a 5 min rinse in 70% ethanol and finally three rinses in MilliQ sterile water for 5 min each time. To promote germination, seeds were first placed on moist sterile filter paper in sterile glass Petri dishes and incubated for 24 h at 18°C (Price 1997, Vasia 2005) (**Fig. 6.4**) and then incubated at room temperature (21±2°C) in the dark. Healthy seedlings 3 days old were transferred to hydroponic tanks by fitting each seedling in a Rockwool plug in one of the punctured holes in the lid; two tanks were used per *Brassica* line per treatment, including uninoculated control. Each hydroponic tank accommodated up to 28 seedlings to generate sufficient root tissue. Plants were fertilised as in Chapter 2.

6.2.3.2.2 Chinese cabbage

For Chinese cabbage, there were minor variations in microorganism removal from that for cabbage: seeds were soaked for 60 min in Mancozeb, treated with 4% NaOCl for 5 min and

rinsed with water five times. Each seedling was sown directly into a damp 35 mm x 35 mm x 40 mm Rockwool seedling plug and left in the dark until germinated for up to 12 days.

Table 6.2: Adapted Hoagland nutrient solution used in the hydroponic system (Taiz and Zeiger 2002; Kong Kaw Wa 2009).

Compound	**Molecular weight**	**Concentration of stock solution (mM)**	**Concentration of stock solution (g L^{-1})**	**Volume of stock solution in final solution (mL L^{-1})**	**Element**	**Final concentration of element**	
						(µM)	**(mg L^{-1})**
Macronutrients							
KNO_3	101.10	1000	101.10	6.0	N	16000	224
$Ca(NO_3)_2.4H_2O$	236.16	1000	236.16	4.0	K	6000	235
$NH_4H_2PO_4$	115.08	1000	115.08	2.0	Ca	4000	160
$MgSO_4.7H_2O$	246.48	1000	246.48	1.0	P	2000	62
					S	1000	32
					Mg	1000	24
Micronutrients							
KCl	74.55	25	1.864	2	Cl	50	1.77
H_3BO_3	61.83	12.5	0.773	2	B	25	0.27
$MnSO_4.H_2O$	169.01	1.0	0.169	2	Mn	2.0	0.11
$ZnSO_4$	287.54	1.0	0.288	2	Zn	2.0	0.13
$CuSO_4.5H_2O$	249.68	0.25	0.062	2	Cu	0.5	0.03
H_2MoO_4 (85% MnO_3)	161.97	0.25	0.040	2	Mo	0.5	0.05
NaFeDTPA (10% Fe)	468.20	64	30.0	0.3	Fe	19.2	0.8

Figure 6.3: Hydroponic system in the controlled growth room with control (left) and treatment (right) tanks using cabbage.

Figure 6.4: Germination of cabbage seeds before transfer to the hydroponic system.

Seedlings were transferred to hydroponic tanks by fitting the Rockwool plugs directly into the perforated lids (Kong Kaw Wa 2009).

6.2.3.3 Inoculation with Plasmodiophora brassicae resting spores

For cabbage, each host plant (susceptible or resistant) seedling was inoculated by pipetting 1 mL of a suspension of 2.5×10^9 spores per mL of a clubroot population [No.1 as a mildly virulent population (ECD 16/02/15) or No.4 as a highly virulent population (ECD 16/02/30)] onto the base of its stem 6 days after germination; suspensions were prepared from galls frozen at -20°C as described previously in Chapter 2. For the control, 1 mL of MilliQ water was applied to each seedling in the control hydroponic tanks instead.

For Chinese cabbage, the concentration of spore suspension was 2×10^7 spores mL^{-1} of clubroot population S, but otherwise the inoculation method was the same.

6.2.4 Histological study of infected host plants

6.2.4.1. Scanning electron microscopy (SEM) (cabbage only)

Inoculated and control pieces of young cabbage roots were selected randomly at 1-30 days after inoculation (dai), cut into pieces approximately 3-5 cm long and fixed in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) at 4°C overnight. Specimens were rinsed four times in the same buffer for 15 min each; this was followed by dehydration in increasing concentrations of ethanol (10%, 25%, 50%, 75%, 90% and 95%) for 15 mins each. The specimens were further dehydrated by two 15 min washes in absolute ethanol (100%, dried over molecular sieve). Dehydrated specimens were critical-point dried using a Ladd Critical Point Drier (Ladd Research Industries Cat. No. 28000). Specimens were mounted on aluminium stubs with double-sided tape and sputter-coated with gold using a sputter coater (Dynavac Mini Coater). The stubs were viewed on a Jeol JSM-840A scanning electron microscope. Results were recorded using ImageSlave for Windows (V.2.11) software and saved as tif image files (**Fig. 6.5**).

Figure 6.5: Apparatus for electron microscopy. (a) Ladd critical point drier, (b) Dynavac sputter coater, (c) Jeol JSM-840A scanning electron microscope.

6.2.4.2 Fixation and staining

6.2.4.2.1 Cabbage

For each treatment, roots of three randomly selected seedlings were harvested daily at 1-15 dai. For each host plant, selected young roots were divided into two groups, the first for tracking infection in the root hairs by light microscopy after clearing and staining and the second for examining sections through roots after fixation and embedding in paraffin wax for light microscopy.

For examination of root hairs, each seedling was gently removed from its Rockwool plug and rinsed free of any remaining adhesive Rockwool using a soft brush. The root was separated from the base of the seedling and fixed individually in an Eppendorf tube (1.5 mL) containing 1:1 (95% acetic acid:95% ethanol) (v/v) to stop further changes in plant and pathogen development. After 12 h, roots were removed from the fixative, washed with demineralized water three times for 5 min, blotted with tissues, placed in 125 ppm aniline blue solution in 50% (v⁄v) acetic acid for 1 min and finally rinsed with tap water for 1 min and stored in 70% ethanol for later examination (Voorrips, 1992; Sharma et al. 2011a). Fifty root hairs per plant were examined for infection and development for each harvest time for each treatment, including controls. Light microscopic observation of stained roots was performed using a Leica DM2500 compound microscope at different magnifications and images of areas of interest saved digitally.

For assessment of cortical colonization, random plants were harvested at 15, 16, 17 and 30 dai. Collected materials were fixed in 5% formaldehyde solution (Sigma) overnight at room temperature, transferred into 70% ethanol and stored at room temperature.

6.2.4.2.2 Chinese cabbage

Roots were harvested at 56 dai, fixed in 4% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0, and stored at room temperature.

6.2.4.3 Embedding and sectioning

Fixed infected roots and galls were cut into 4 mm diameter pieces if larger and placed into tissue cassettes. Tissues were processed using a Leica ASP200S tissue processor programmed for standard tissues based on the manufacturer's protocol (**Table 6.3**). Tissues were embedded in molten paraffin wax with the surface to be cut facing down in the block (Ruzin 1999) using a Shandon Histocentre 3 (Thermo Scientific) and allowed to solidify (**Fig. 6.6**).

The embedded tissues were sectioned at 4-5 µm thick, floated on a water bath and fixed on glass slides at 50ºC for 1 h (Johansen, 1940; Ma et al. 1993). Sections were photographed on a Leica DM2500 compound microscope and images recorded as before.

Table 6.3: The standard processing program used in the Leica ASP 200S tissue processor.

Reagents	**Station**	**Duration (hh:mm)**	**Temperature (°C)**	**Drain (sec)**	**P/V**
Neutral buffered formalin	1	01:00	N/A	140	V
Ethanol 70%	2	00:45	N/A	120	V
Ethanol 90%	3	00:45	N/A	120	V
Ethanol absolute	5	00:45	N/A	120	V
Ethanol absolute	6	01:00	N/A	120	V
Ethanol absolute	7	01.00	N/A	120	V
Xylene	8	00:45	N/A	120	V
Xylene	10	01:00	N/A	120	V
Xylene	9	01:00	N/A	140	V
Paraffin wax	Wax (I)	01:00	62	140	P/V
Paraffin wax	Wax (II)	00:10	62	140	P/V
Paraffin wax	Wax (III)	01:00	62	140	P/V

P/V - P= Pressure; V= Vacuum

Figure 6.6: Embedding samples in paraffin wax (left) and cutting sections (right).

6.2.4.4 Staining of tissue sections (permanent slides)

Sections were rehydrated and stained in glass Coplin jars. Wax was removed and sections were rehydrated through an EtOH series. Sections were stained with Safranin O and counterstained with Fast Green and mounted in DPX before being examined with a Leica DM2500 compound microscope and images recorded as before.

The dehydration, staining and mounting procedures used were as follows. Sections were

- dewaxed with three 5 min washes in 100% xylene
- rehydrated through an EtOH series (100%, 100%, 95%, 85%, 70%) for 5 min each
- stained for 2 h in 1% (w/v) Safranin O solution
- rinsed with ddH_20 (Milli-Q water) twice for 5 min each with gentle agitation
- dehydrated for 10 s in 95% EtOH plus 0.5% HCl
- washed for 10 s - 1 min in 95% EtOH plus four drops NH_4OH per 100 mL (no longer time or it would destain the tissue completely)
- dipped for about 10 s in 100% EtOH to finish dehydration
- counterstained for 5-15 s in 0.05% (w/v) Fast Green (the best time for staining with Fast Green not being more than 5 s to prevent over-staining). Methylene blue was used instead of Fast Green to see if the dye stained both the plant and pathogen and so some images are stained blue instead.
- rinsed free from excess Fast Green with “used” (diluted) clearing solution
- washed in clearing solution by dipping for 10 s
- dipped in xylene plus 2-3 drops of 100% EtOH to remove clearing solution
- cleared in xylene (three washes of xylene for 5 min each
- mounted in DPX and stored at room temperature.

6.2.5. Data analysis

For cabbage, the timings of each stage of infection in the root hairs were compared. In older stages, the numbers of uninfected cells, and of infected cells with resting spores and with plasmodia were counted for each treatment as in Donald and Porter (2004a). Similar counts were made for Chinese cabbage. A total of 150 separate fields of view was counted for each of the three variables for each species of plant inoculated with each population (n= 2700). χ^2 statistics were calculated to test the null hypothesis that the proportions of each type of cell in the cortical cells of the roots/galls were equal for each type of plant host for each population.

6.3 Results

6.3.1 Cabbage (B. oleracea)

6.3.1.1 Macroscopic symptoms in cabbage

Clubroot symptoms developed differentially in the hydroponic system, at least in susceptible host plants (**Fig. 6.7**), as described in detail below. Clubroot incidence was greater in susceptible cabbage plants and there was no galling in resistant plants.

6.3.1.1.1 Susceptible plants – macroscopic symptoms on roots

By 25 dai, plants infected with both populations showed normal large galls on the roots (**Fig. 6.7a**). Those infected with population No. 4 also showed infection of the hypocotyl and lower stem (**Fig. 6.7b**).

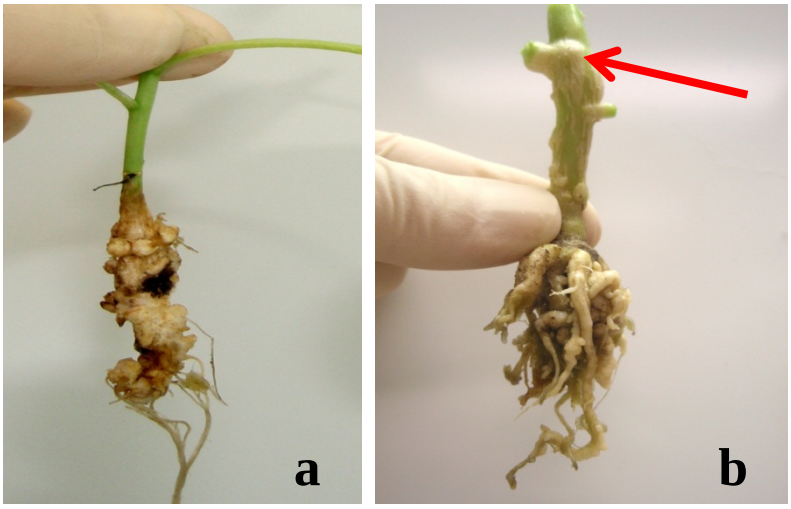

Figure 6.7: Susceptible cabbage plants infected with populations of *Plasmodiophora brassicae*, (a) Infected host roots with population No.1 at 25 dai, (b) root infected with population No.4 at 25 dai; the infection reached the stem (arrow).

Susceptible hosts showed clear differences in amount, shape and appearance of the galls in the roots between the mildly virulent population No. 1 and the highly virulent population No. 4. Swelling of the hypocotyl was the first clear symptom of disease and was observed 3 days earlier (after 12 dai) with population No. 4 than with population No.1. Galling with population No.1 was later and less than with population No. 4 at each collection time (**Figs 6.8-6.10**). At 20 days the roots had formed easily visible galls with population No. 4 whereas galls were barely visible with population No. 1.

Figure 6.8: Cabbage roots in hydroponic system infected with populations of *Plasmodiophora brassicae*, at 15 dai (a, c) and 20 dai (b, d) in susceptible plants infected with population No. 1 (a, b) and population No. 4 (c, d).

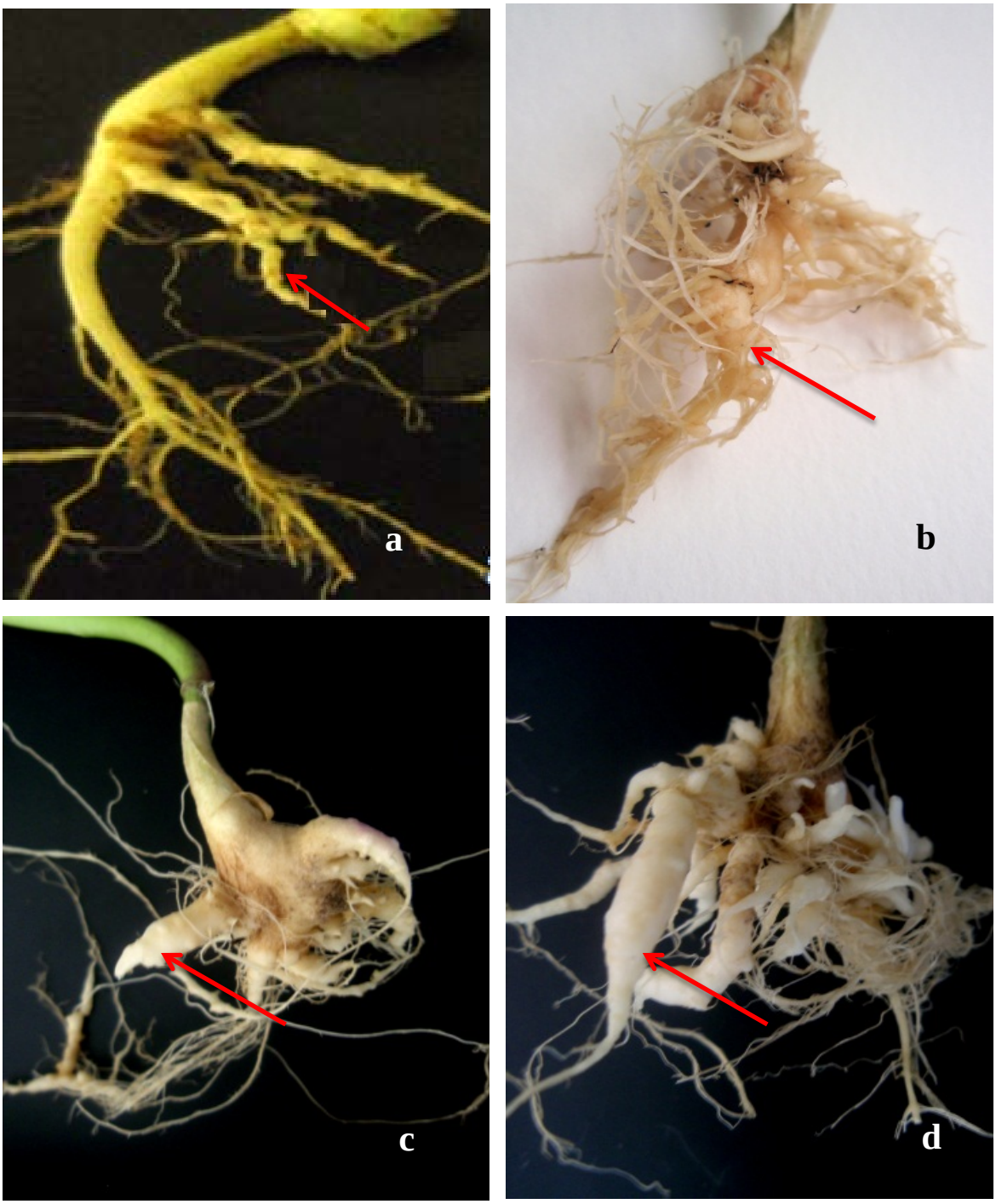

Figure 6.9: Susceptible cabbage infected with population No.1 of *Plasmodiophora brassicae* (a) 18 dai; (b) 20 dai; (c) 25 dai and (d) 28 dai. (Arrows point to the galls or swellings on the root system).

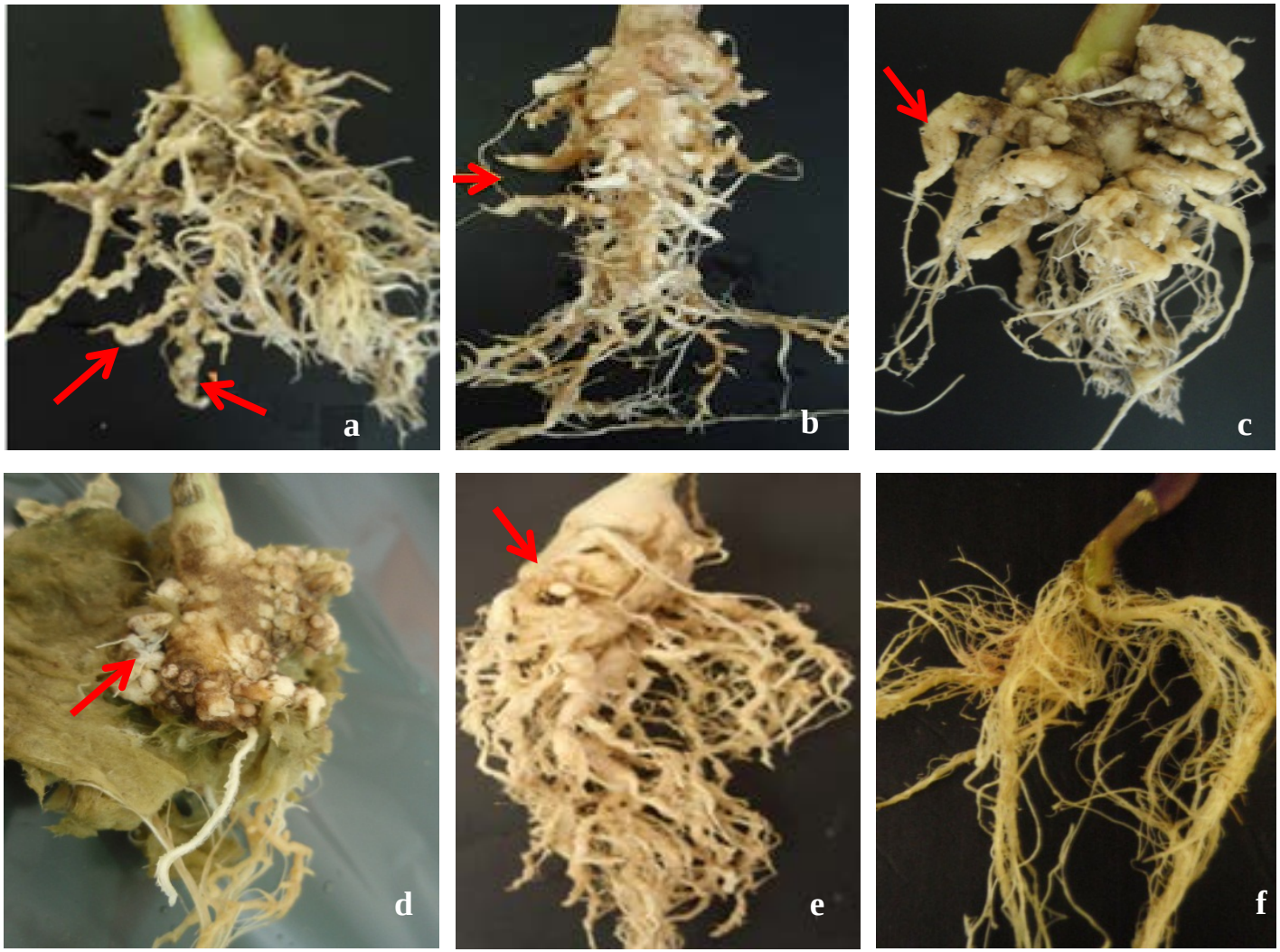

Figure 6.10:Cabbage plants infected with population No.4 of *Plasmodiophora brassicae*, (a) 18 dai; (b) 20 dai; (c) 25dai; (d,e) 28 dai ; and (f) control plants (uninoculated). Arrows point to the galls or swellings on the root system.

6.3.1.1.2 Resistant plants – macroscopic symptoms on roots

No macroscopically visible galls were present on the root systems of either treatment (**Fig. 6.11**).

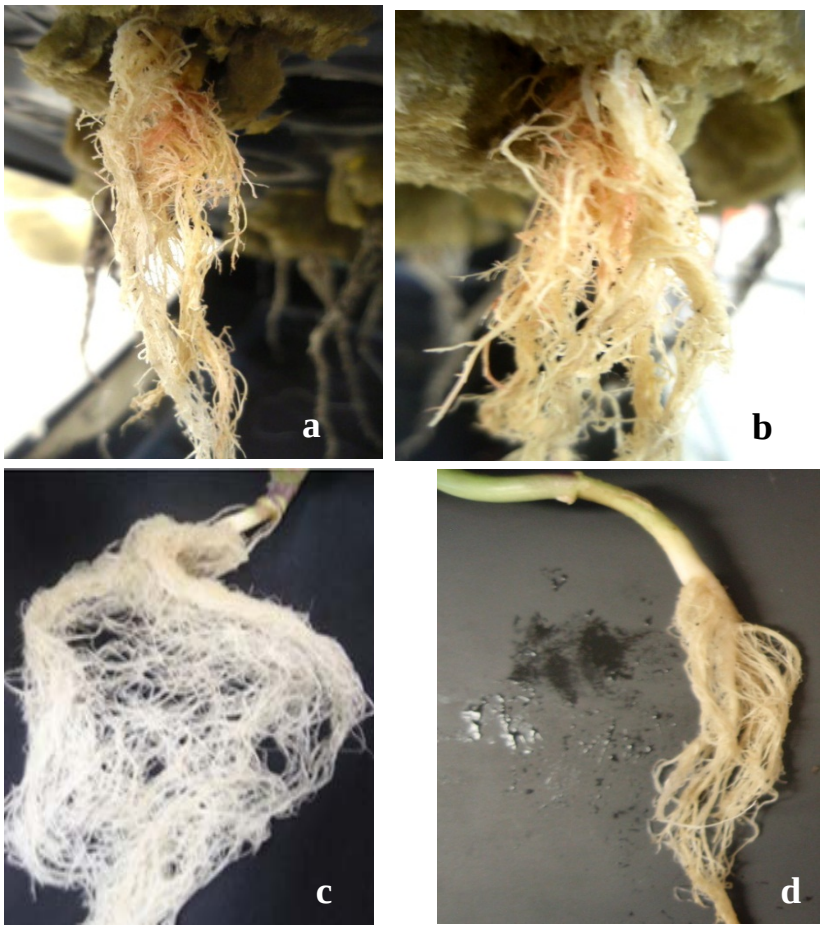

Figure 6.11: Resistant cabbage plants at 20 and 30 dai respectively with *Plasmodiophora brassica,* (a,c) with population No.1, (b,d) with population No. 4.

6.3.1.1.3 Changes in the appearance of infected plants

Plant development generally was affected in inoculated plants of susceptible plants compared with uninoculated (control) plants and resistant host plants, though wilting, chlorosis and senescence was noted with both types of cabbage plants were severely stunted (**Fig. 6.12**). In leaves, reduction in size, distortion and wrinkling was noted (**Fig. 6.13**).

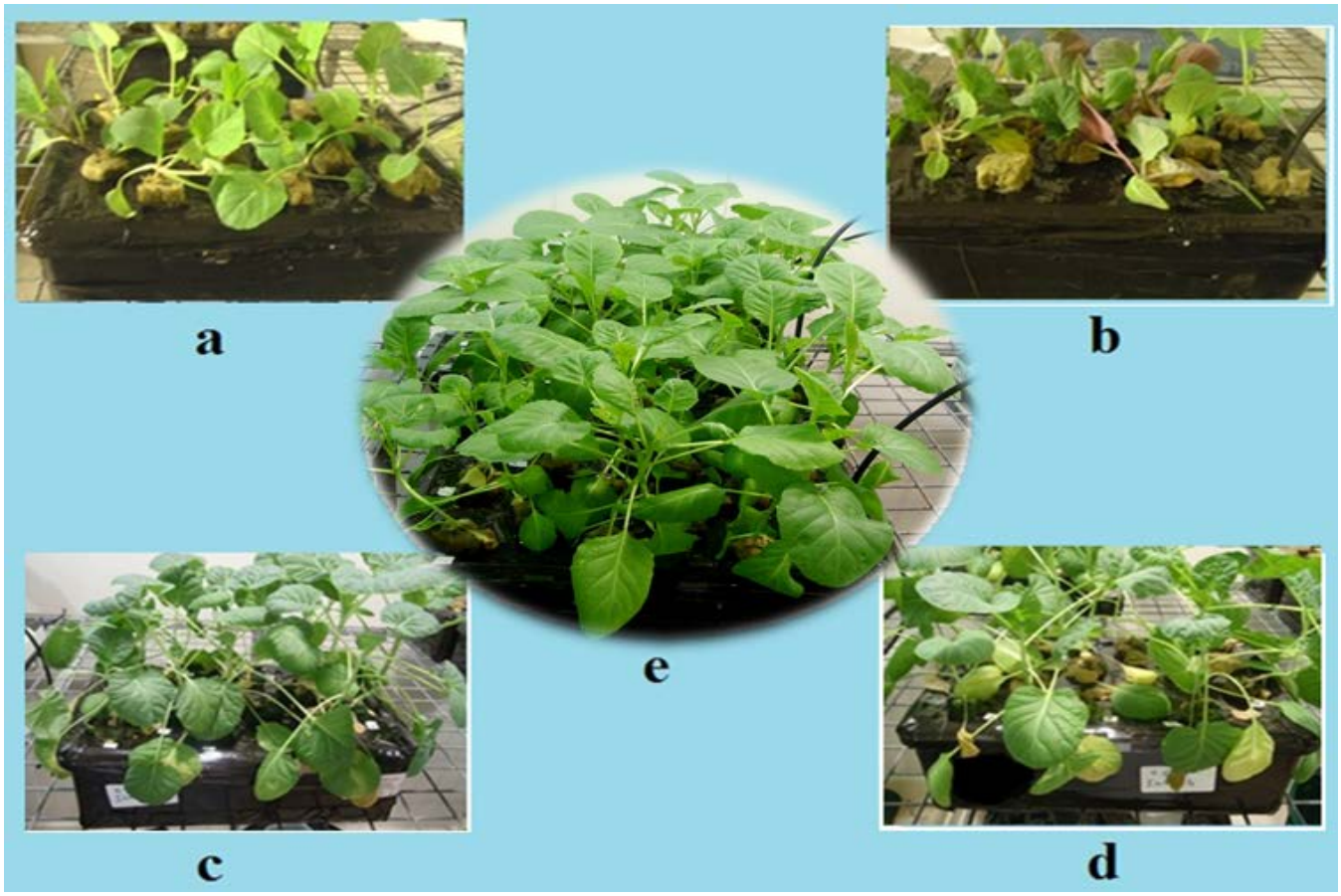

Figure 6.12: Appearance of cabbage plants with and without inoculation with *Plasmodiophora brassicae* growing in hydroponic tanks at 20 (dai), (a + b) susceptible hosts, (c+ d) resistant host plants and (e) control.

Figure 6.13: Symptoms on leaves of cabbage plants inoculated with *Plasmodiophora brassicae* at 25 dai with population No. 4 of *Plasmodiophora brassicae*. Note changes in leaves from their normal shape and the occurrence of wrinkling.

6.3.1.2 Scanning electron microscopy of cabbage roots

6.3.1.2.1 Low virulence pathotype (population No.1)

No resting spores were observed at 1 dai, but at 2-15 dai abundant resting spores were observed on root hairs for both susceptible and resistant host plants (**Fig. 6.14**). The resting spores were globose-ovoid and about 2-3 µm diameter, with a spiny surface (**Fig. 6.14e,f**). Resting spores seemed more frequent on susceptible plants but no quantitative data were collected. At 21 dai, resting spores were still present on root hairs of susceptible plants but not on those of resistant plants (**Fig. 6.15**). This difference persisted until 25-28 dai (**Fig. 6.16**). Resting spores were never observed on uninoculated control plants (**Fig. 6.20**).

6.3.1.2.2 High virulence pathotype (population No.4)

Resting spores were present on root hairs from 1 dai and continued to be present throughout 2-12 dai (**Fig. 6.17**). Resting spores resembled those of population No. 1 (**Fig.6. 17e,f**). There again appeared to be fewer resting spores on resistant than susceptible host plants but no quantitative data were collected. Resting spores continued to be present on both types of root until 15 dai (**Fig. 6.18**) but thereafter they were observed only on susceptible plants (**Fig. 6.19**). Resting spores were never observed on uninoculated control plants (**Fig. 6.20**).

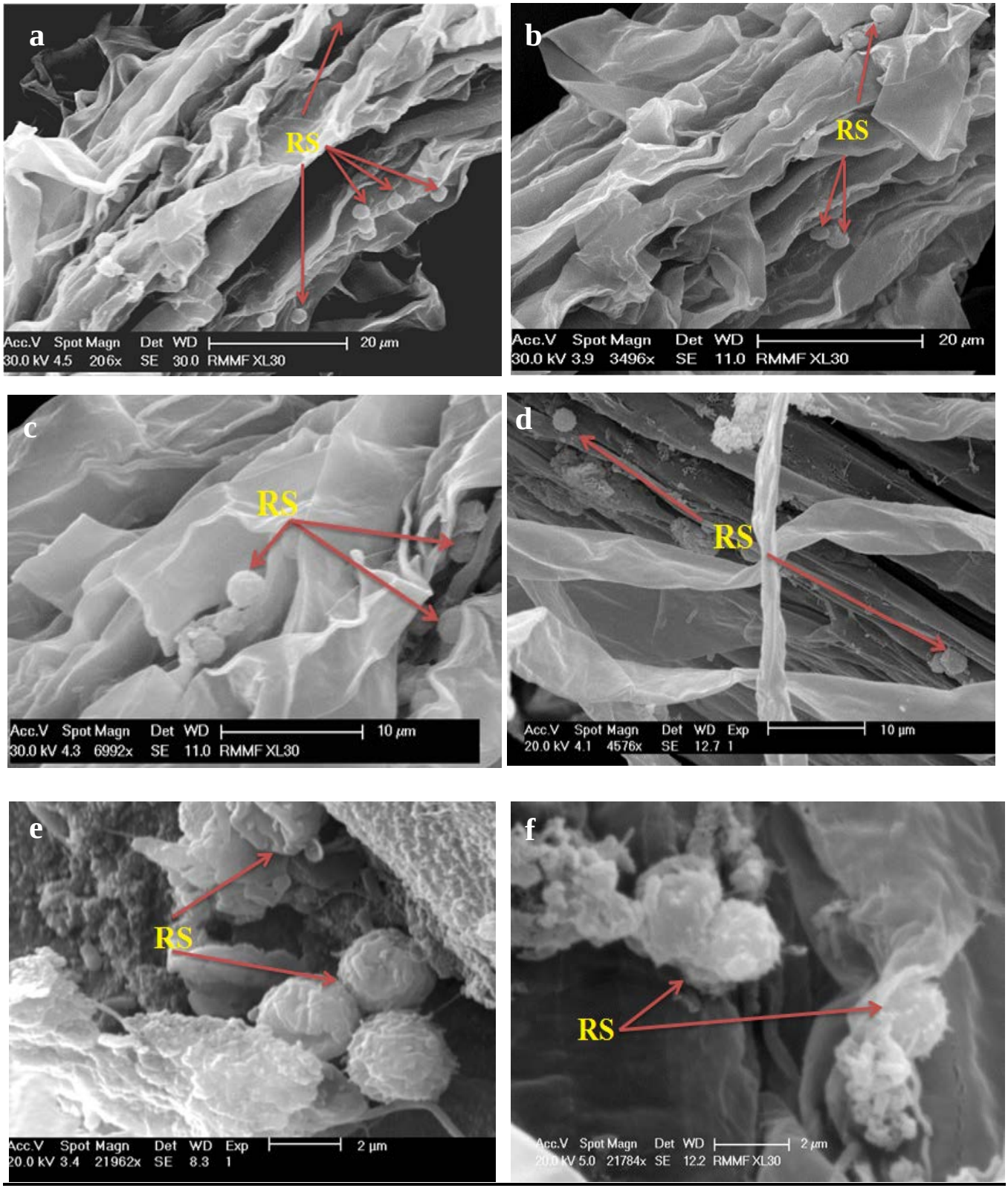

Figure 6.14: Scanning electron micrographs of susceptible (a,c,e) and resistant (b,d,f) cabbage roots showing resting spores of population No. 1 on the surfaces of root hairs at 1-15 dai.

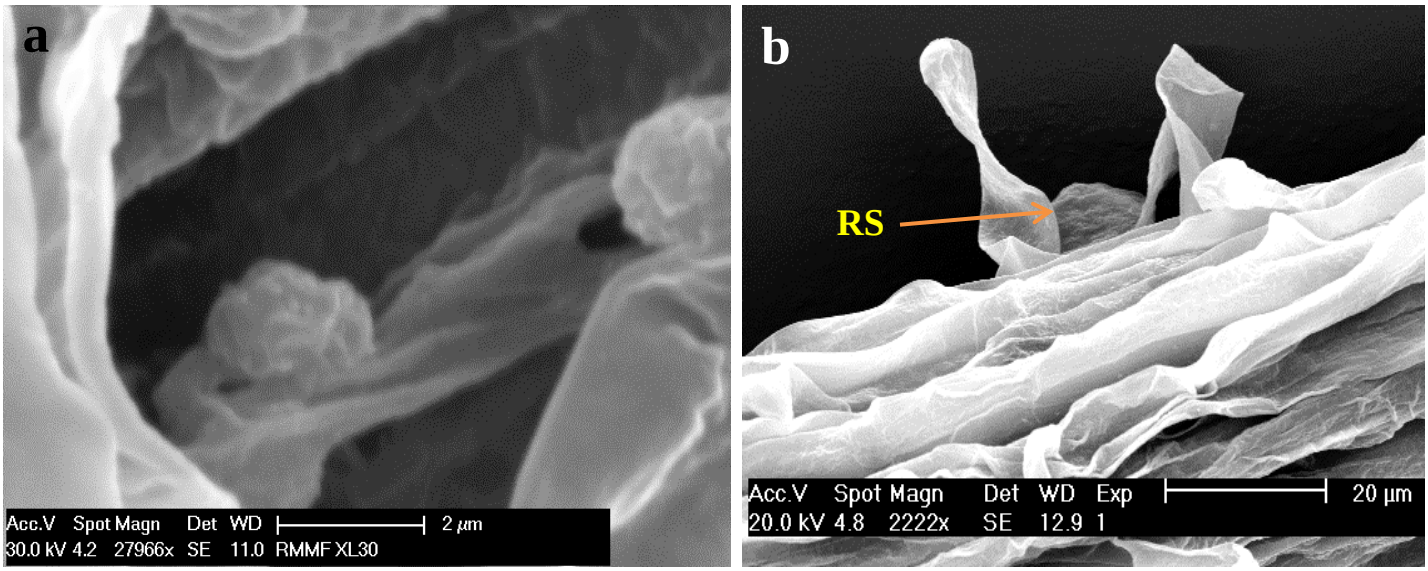

Figure 6.15: Scanning electron micrographs of susceptible (a) and resistant (b).cabbage roots, showing resting spores of population No. 1 on the surface of root hairs at 21 dai.

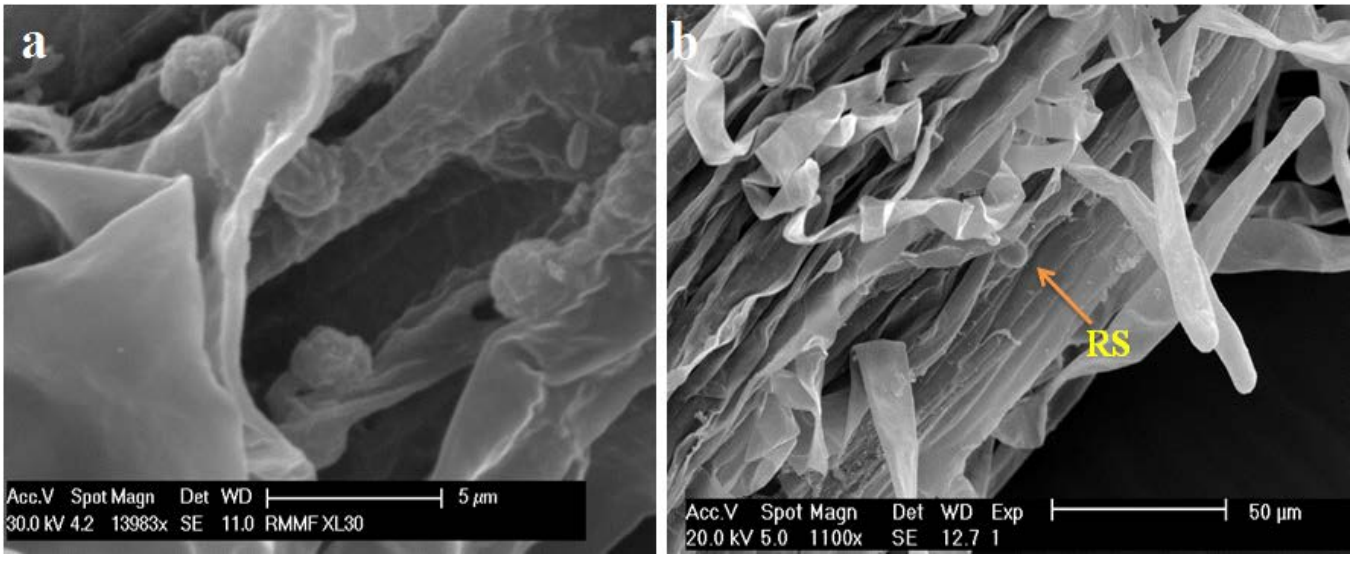

Figure 6.16: Scanning electron micrographs of susceptible (a) and resistant (b) cabbage roots, showing resting spores of population No. 1 on the surface of root hairs at 25-28 dai.

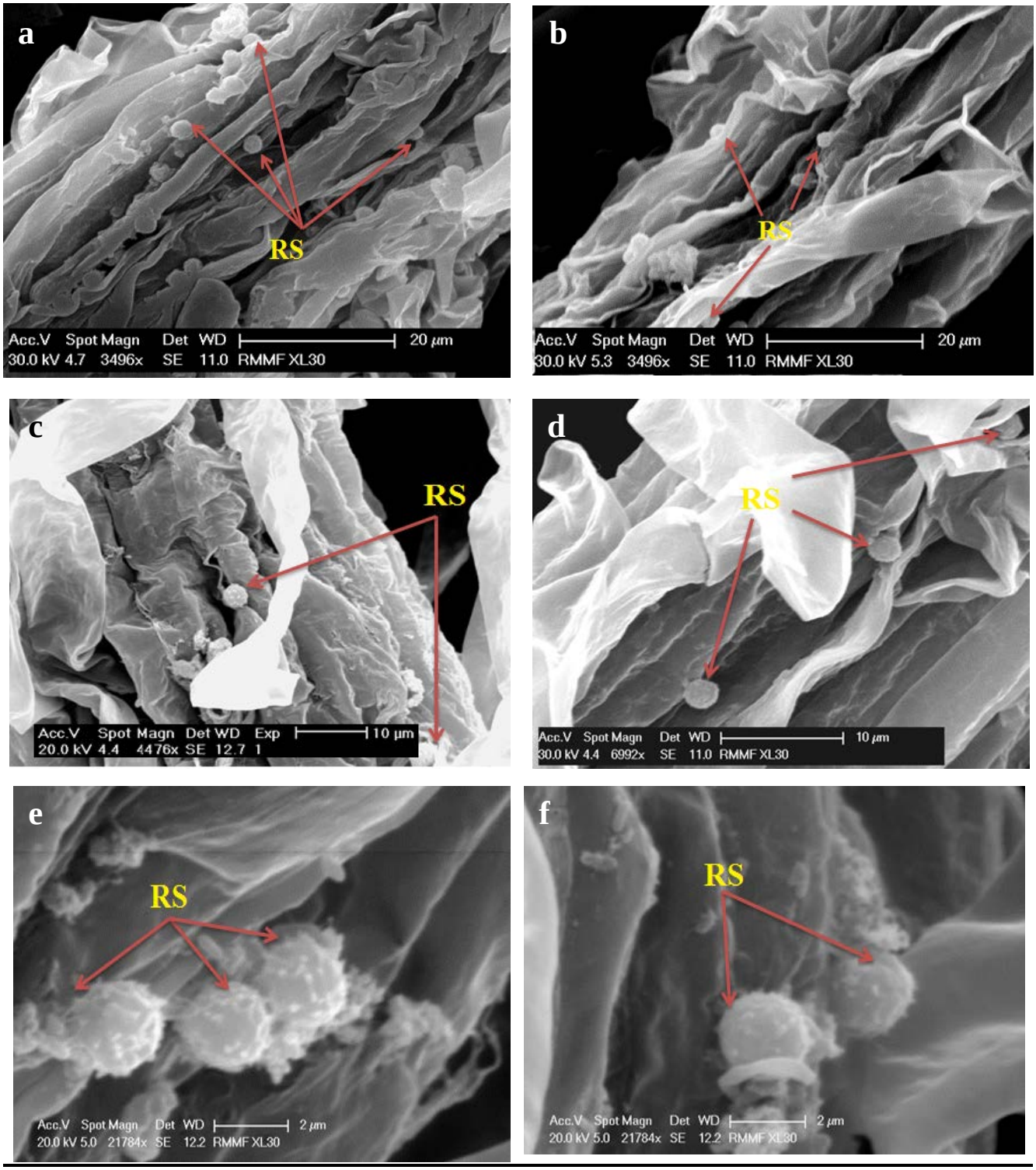

Figure 6.17: Scanning electron micrographs of susceptible (a,c,e) and resistant (b,d,f) cabbage roots showing resting spores of population No. 4 on the surfaces of root hairs at 2-12 dai.

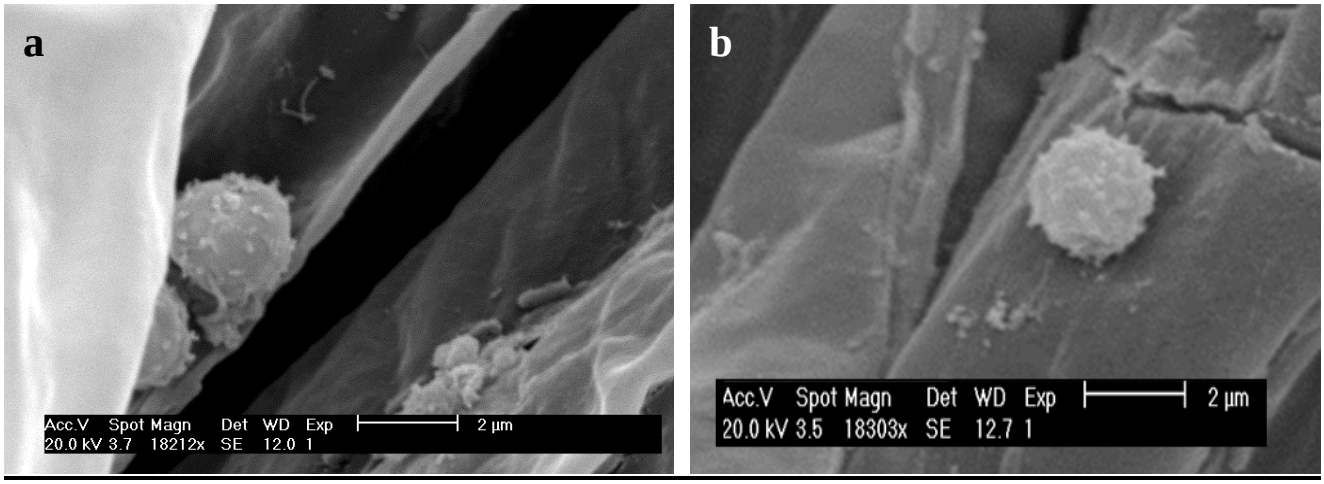

Figure 6.18: Scanning electron micrographs of susceptible (a) and resistant (b) cabbage roots showing resting spores of population No. 4 on the surfaces of root hairs at 13-15 dai.

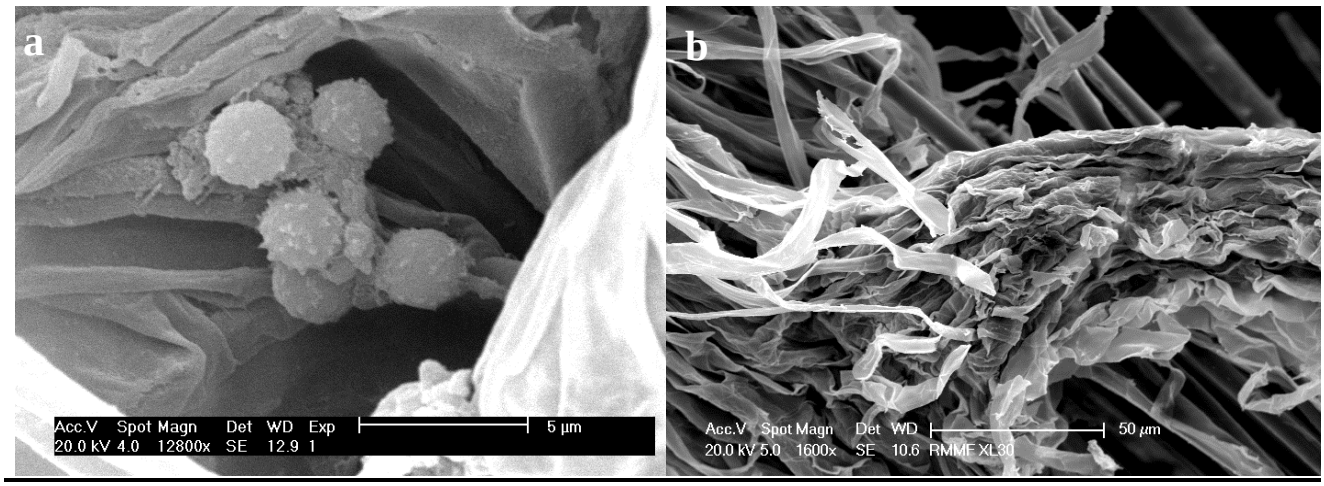

Figure 6.19: Scanning electron micrographs of susceptible (a) and resistant (b) cabbage roots showing resting spores of population No. 4 on only the surfaces of root hairs of susceptible cabbage roots (a) at 16-28 dai. Note lack of resting spores attached to the root surfaces of resistant host plants.

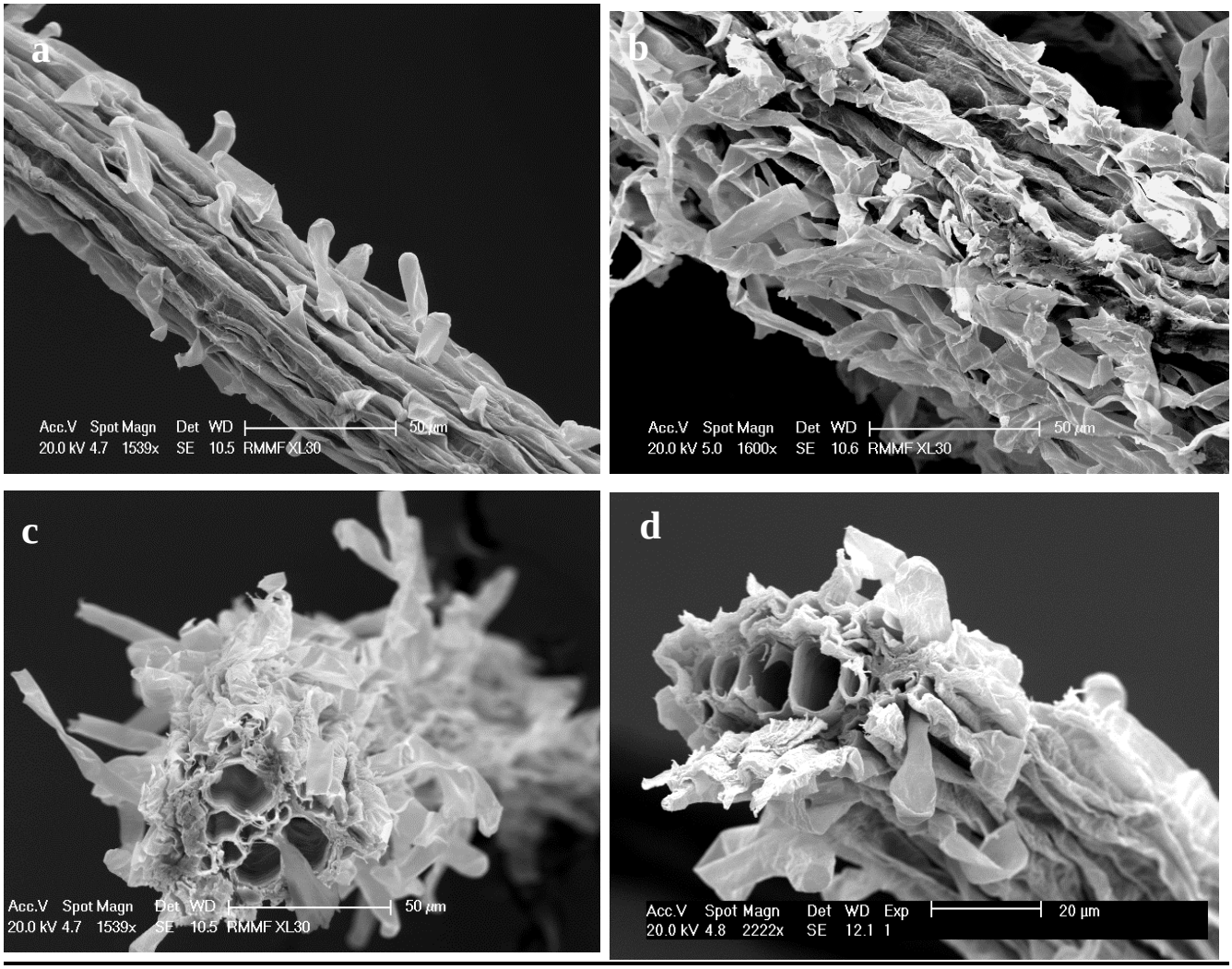

Figure 6.20: Scanning electron micrographs for uninoculated host plants (control) showing the intact surface of root hairs. Susceptible (a,c), resistant (b,d).

6.3.1.3 Root hair infection by light microscopy

All stages of infection were observed in root hairs using plants from the hydroponic system.

6.3.1.3.1 Low virulence pathotype (population No. 1)

All stages of primary infection occurred in root hairs of both susceptible and resistant plants (**Table 6.4**). Infection of root hairs was initiated at 1-4 dai when primary zoospores from germinated resting spores (**Figs 6.21, 6.27**) encysted on the root hair surface and produced primary plasmodia inside the root hairs at 4-8 dai (**Figs 6.22, 6.28**). Large multinucleate plasmodia (**Figs 6.23, 6.29**) differentiated into many zoosporangia at 7-12 dai (**Figs 6.24, 6.30**), which released secondary zoospores, leaving empty zoosporangia inside the root hairs at 15-18 dai (**Figs 6.24, 6.30**). Secondary plasmodia and amoeboid forms of *P. brassicae* were noted within cortical cells at 17-30 dai (**Figs 6.26, 6.32**). Between susceptible and resistant plants, all timings of each stage overlapped and there was no difference in the appearance of any stage.

Table 6.4: Stages in infection of susceptible and resistant cabbage by low (mildly) virulent *Plasmodiophora brassicae* population No. 1.

Days after inoculation (dai)		**Stages observed in root hairs**	**Figure**	
Susceptible	**Resistant**		**Susceptible**	**Resistant**
1-4	2-3	Primary zoospore encysted on root hairs.	6.20	6.26
5-8	4-6	Young primary plasmodia in root hairs.	6.21	6.27
9-12	7-12	Large differentiating primary plasmodia and immature zoosporangia.	6.22	6.28
13-14	13-16	Fully differentiated zoosporangia.	6.23	6.29
15-17	17-18	Zoospore release from root hairs evidenced by empty zoosporangia.	6.24	6.30
17-30	19-30	Secondary plasmodia and amoeboid forms.	6.25	6.31

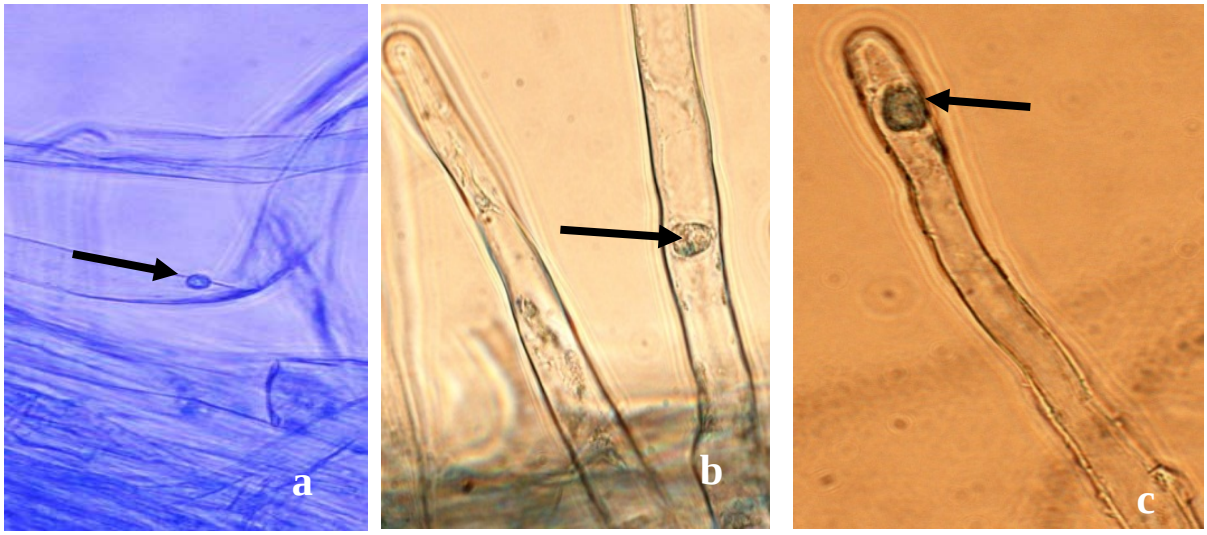

Figure 6.21: Stages in root hair infection of susceptible cabbage by population No. 1 at 1-4 dai, showing (arrows) primary zoospores encysted on root hairs. Magnification:a x600, b-c x1000.

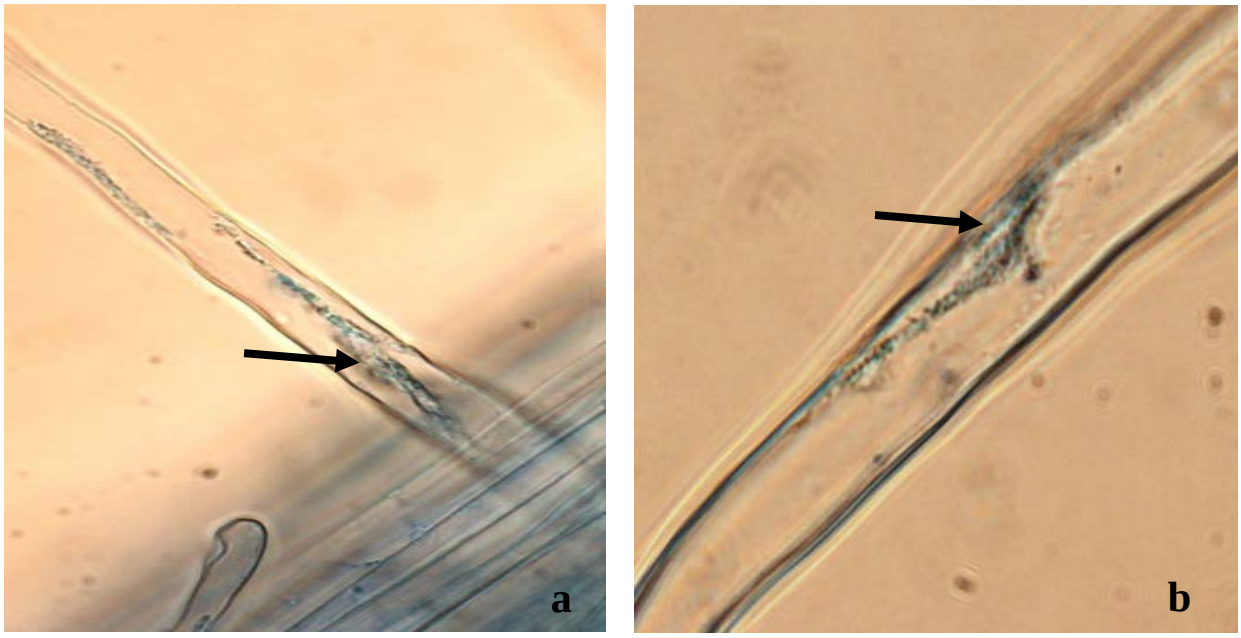

Figure 6.22: Stages in root hair infection of susceptible cabbage by population No. 1 at 5-8 dai, showing young plasmodia (arrows) in root hairs. Magnification: a-bx1000.

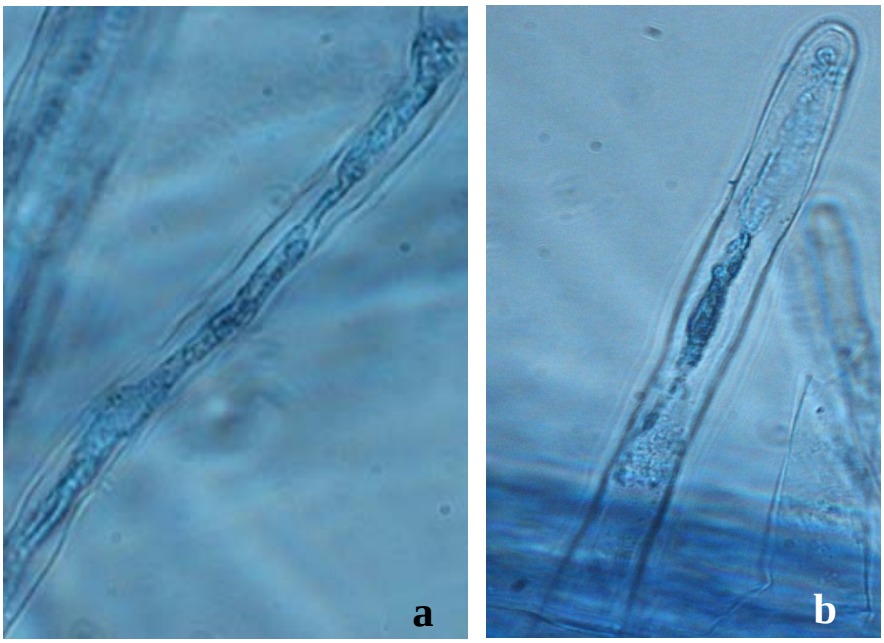

Figure 6.23: Stages in root hair infection of susceptible cabbage by population No. 1 at 9-12 dai, showing large, differentiating plasmodium (a) and immature zoosporangia (b) in root hairs. Magnification:a-b x1000.

Figure 6.24: Stages in root hair infection of susceptible cabbage by population No. 1 at 13-14 dai, showing fully differentiated zoosporangia in root hair. Magnification: x1000.

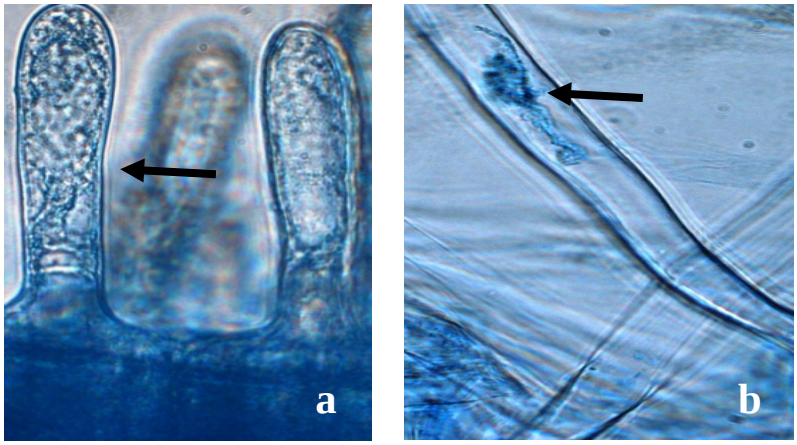

Figure 6.25: Stages in root hair infection of susceptible cabbage by population No. 1 at 15-17 dai, showing differentiated zoosporangia (a) and evidence of zoospore release (empty zoosporangia) (b) (arrows). Magnification: x1600.

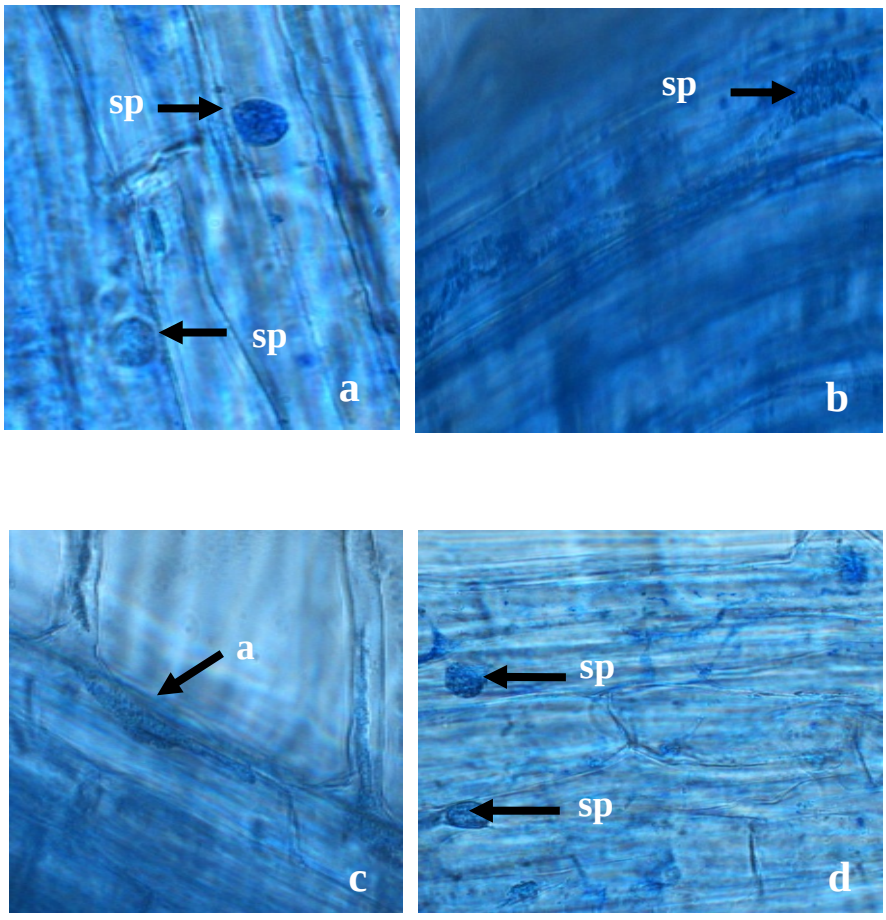

Figure 6.26: Stages in infection of susceptible cabbage by population No. 1 at 17-30 dai, showing secondary plasmodia (sp) and amoeboid (a) forms (arrows). Magnification:a-b x1000.

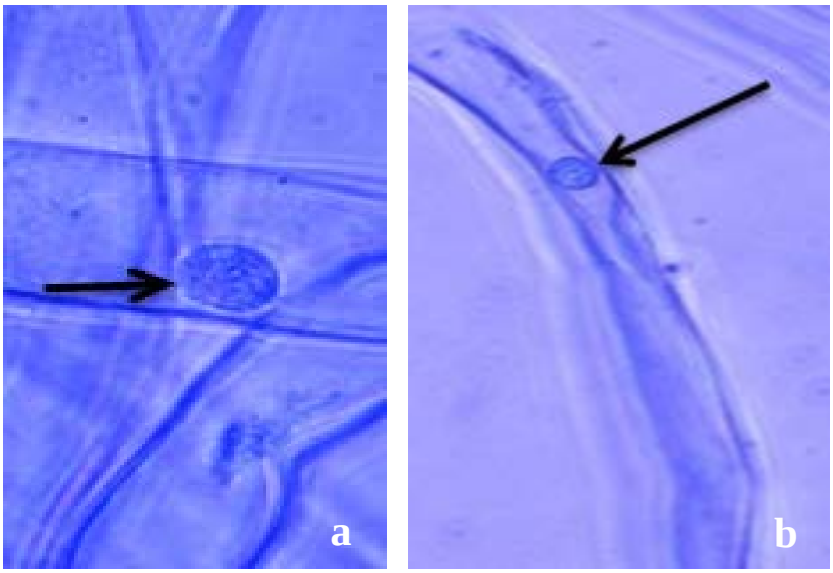

Figure 6.27: Stages in infection of resistant cabbage by population No. 1 at 2-3 dai, showing primary zoospores encysted on root hairs (arrows). Magnification: a-b x1600.

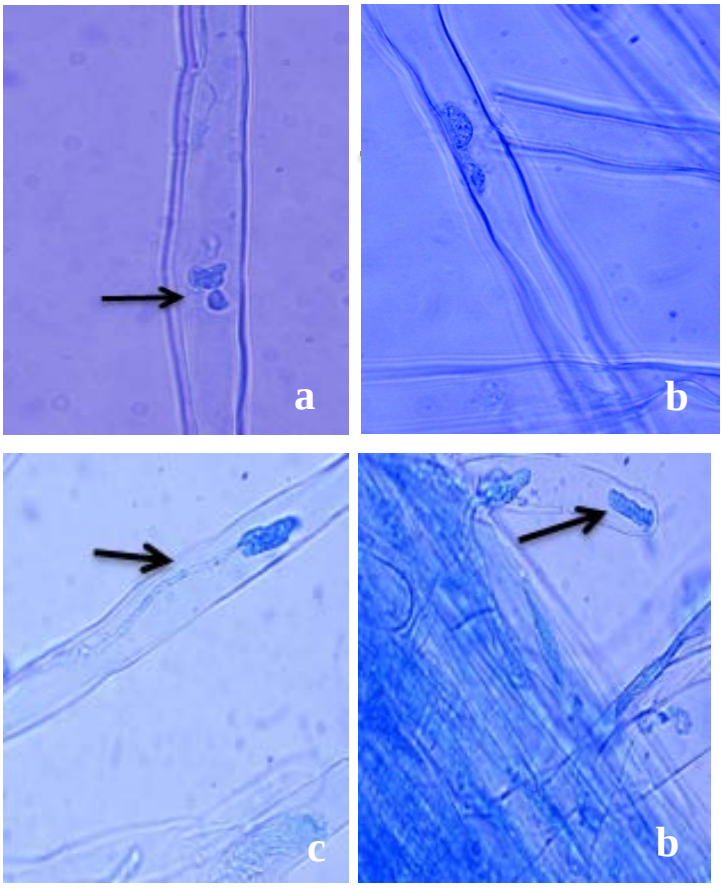

Figure 6.28: Stages in infection of resistant cabbage by population No. 1 at 4-6 dai, showing young primary plasmodia (arrows) in root hairs. Magnification:a-c x600.

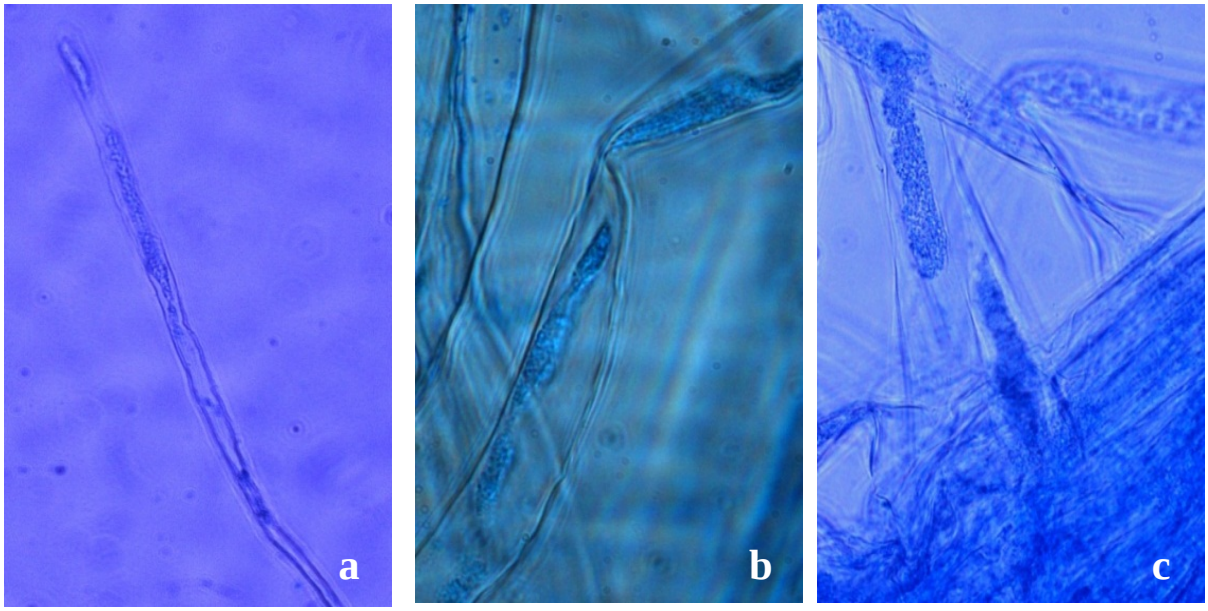

Figure 6.29: Stages in infection of resistant cabbage by population No. 1 at 7-12 dai, showing large differentiating plasmodia (left+center) and immature zoosporangia (right) in root hairs. Magnification:a x600, b-c x1600.

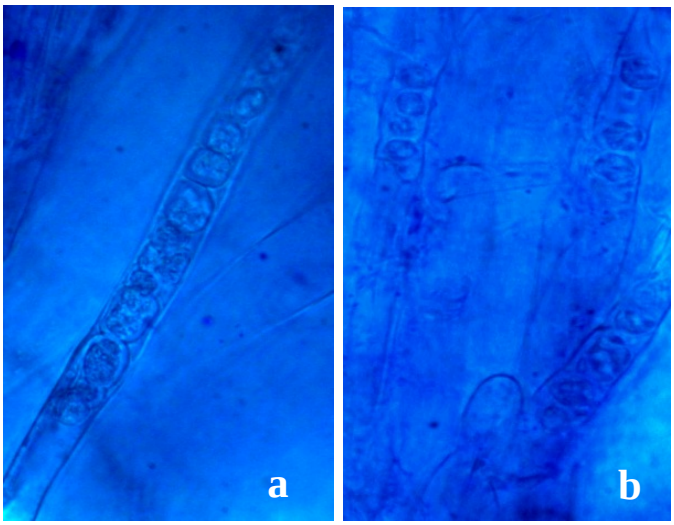

Figure 6.30: Stages in infection of resistant cabbage by population No. 1 at 13-16 dai, showing fully differentiated zoosporangia in root hairs (a-b). Magnification: a-b x 1600.

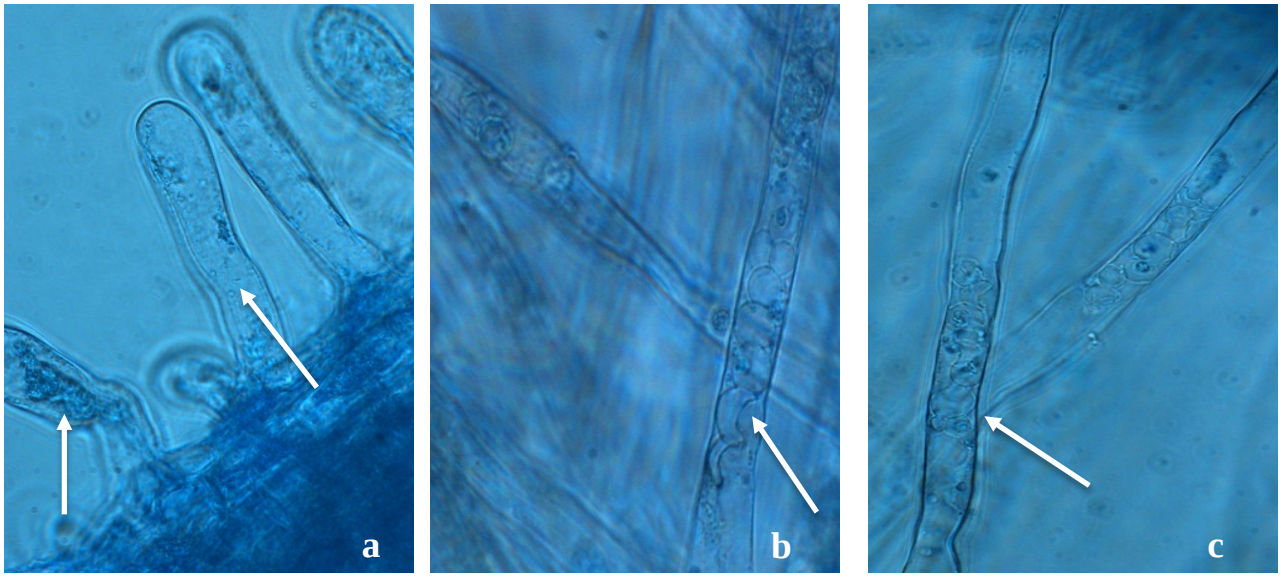

Figure 6.31: Stages in infection of resistant cabbage by population No. 1 at 17-18 dai, showing differentiated zoosporangia and evidence of zoospore release (empty zoosporangia) (b) (arrows). Magnification:a-c x1000.

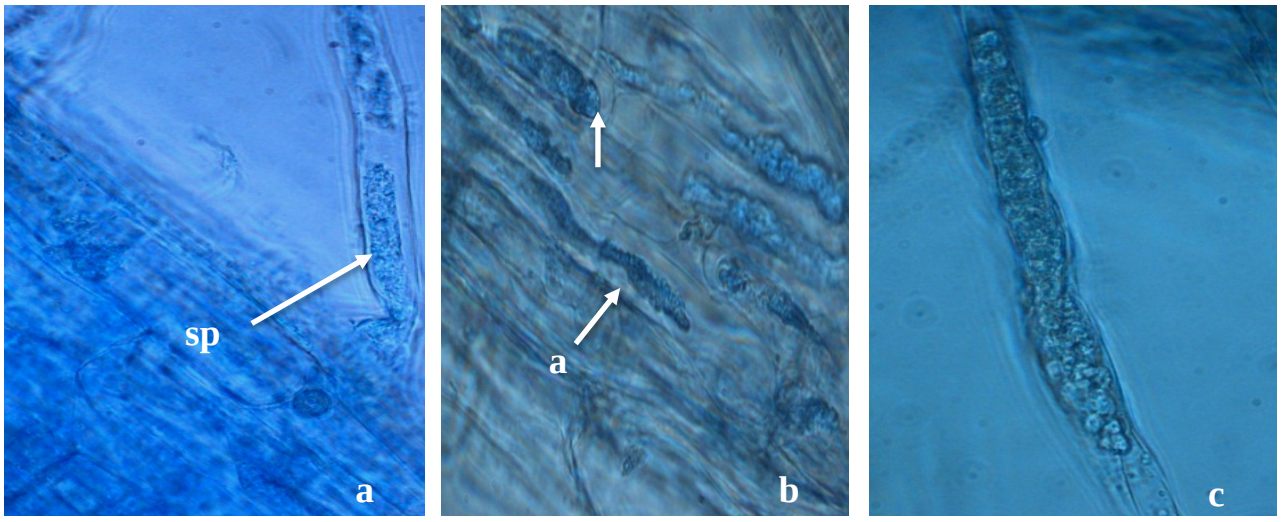

Figure 6.32: Stages in infection of resistant cabbage by population No. 1 at 19-30 dai, showing secondary plasmodia (sp) and amoeboid (a) forms. Magnification: a x1000, b-c x 1600.

6.3.1.3.2 High virulence pathotype (population No. 4)

All stages of infection were observed in both susceptible and resistant plants (**Table 6.5**). Primary zoospores encysted on root hairs at 1 dai (**Figs 6.33, 6.38**). Young primary plasmodia were present at 2-7 dai (**Figs 6.34, 6.39**) and large multinucleate plasmodia and immature zoosporangia were noted at 5-9 dai (**Figs 6.35, 6.40**). Mature, fully differentiated zoosporangia were observed at 9-16 dai (**Figs 6.36, 6.41**) and secondary zoospore release was suggested by empty zoosporangia (**Figs 6.37, 6.42**) and secondary plasmodia (**Figs 6.37, 6.43**) at 17-30 dai. The ranges in timings of the various stages overlapped between susceptible and resistant plants (**Table 6.16**) and there was no difference in their appearance at any stage.

Table 6.5: Stages in infection of susceptible and resistant cabbage by highly virulent *Plasmodiophora brassicae* population No. 4.

Days after inoculation (dai)		**Stages observed in root hairs**	**Figure**	
Susceptible	**Resistant**		**Susceptible**	**Resistant**
1	1	Primary zoospores encysted on root hairs.	6.32	6.37
2-7	2-4	Young primary plasmodia in root hairs.	6.33	6.38
8	5-9	Large differentiating primary plasmodia and immature zoosporangia.	6.34	6.39
9-16	10-14	Fully differentiated zoosporangia.	6.35	6.40
17-30	18-30	Zoospore release from root hairs evidenced by empty zoosporangia.	6.36	6.41
9-30	18-30	Secondary plasmodia and amoeboid forms.	6.36	6.42

Figure 6.33: Stages in infection of susceptible cabbage by population No. 4 at 1 dai, showing primary zoospore encysted on root hair (arrows). Magnification: x1000.

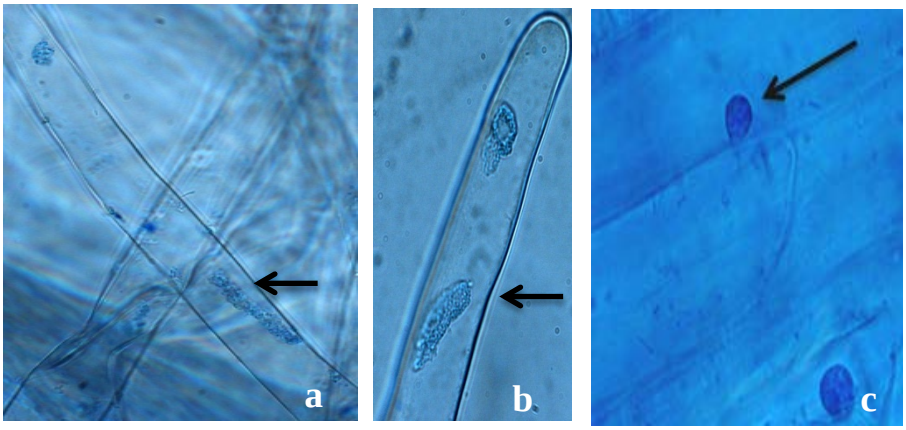

Figure 6.34: Stages in infection of susceptible cabbage by population No. 4 at 2-7 dai, showing young primary plasmodia (arrows) in root hairs and penetration of root hair cell wall. Magnification: a x1000, b-c 1600.

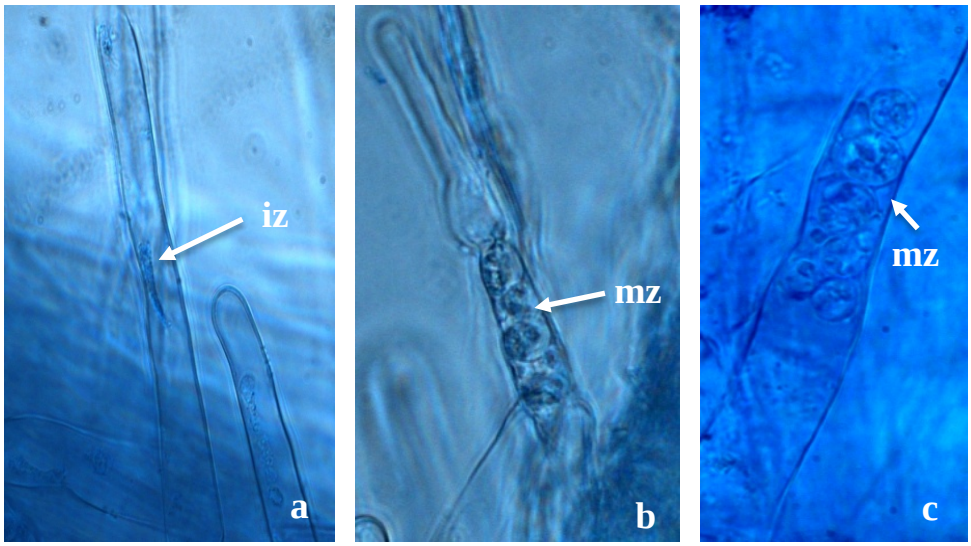

Figure 6.35: Stages in infection of susceptible cabbage by population No. 4 at 8 dai, showing immature (iz) and mature, fully differentiated (mz) zoosporangia in root hairs. Magnification: a x1000, b x1000, c x1600.

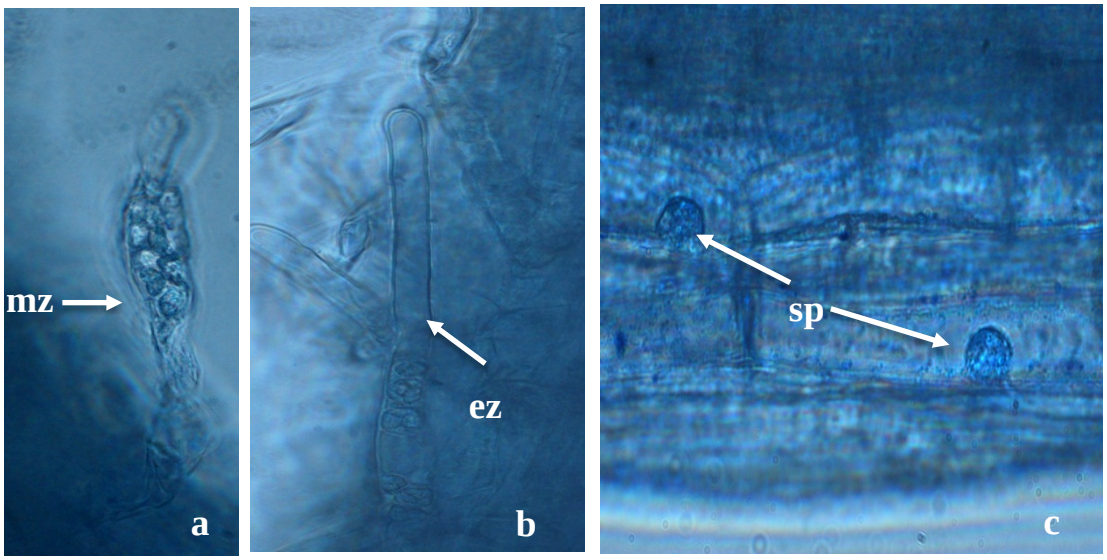

Figure 6.36: Stages in infection of susceptible cabbage by population No. 4 at 9-16 dai, showing mature, fully differentiated zoosporangia (mz) and secondary plasmodium (sp) in the cortex. Magnification: a-b x1000, c x1600.

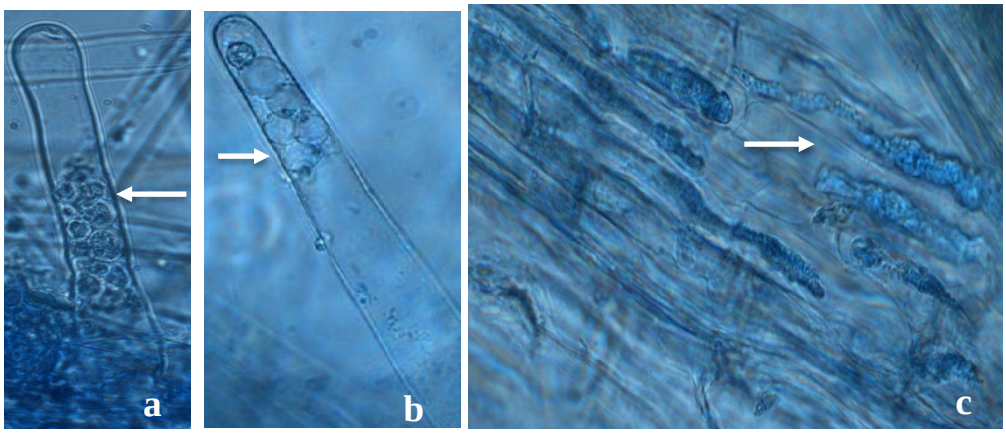

Figure 6.37: Stages in infection of susceptible cabbage by population No. 4 at 17-30 dai, showing zoospores in mature zoosporangia (left), empty zoosporangia (centre) and a cluster of secondary plasmodia (arrow). Magnification: a-c x 1600.

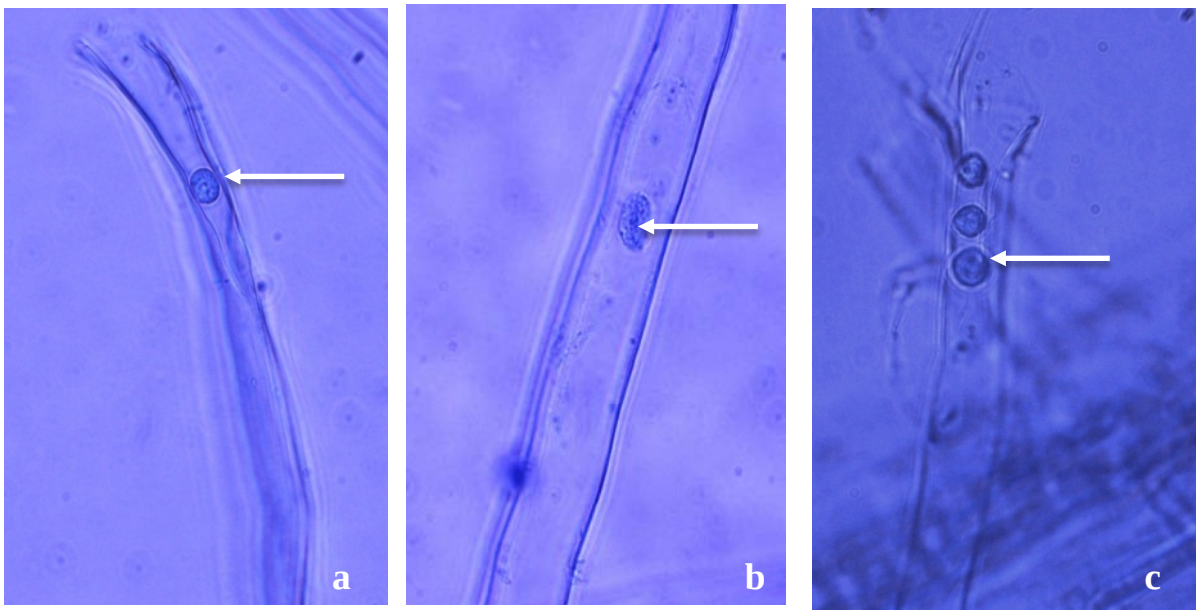

Figure 6.38: Stages in infection of resistant cabbage by population No. 4 at 1 dai, showing primary zoospores encysted on root hairs (arrows). Magnification: a-c x1000.

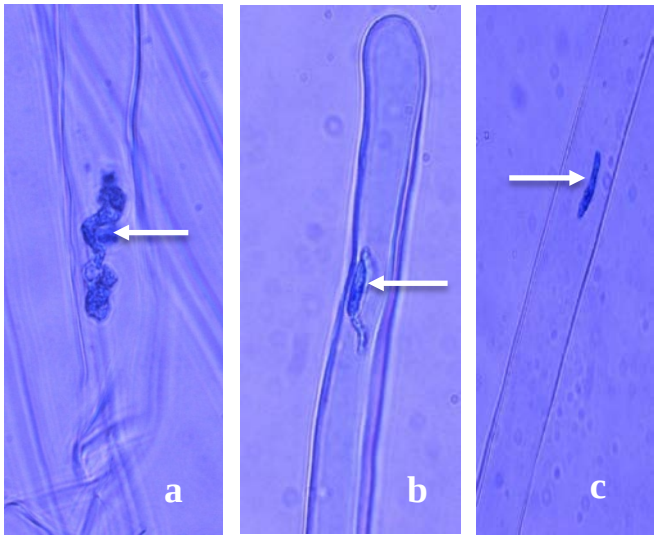

Figure 6.39: Stages in infection of resistant cabbage by population No. 4 at 2-4 dai, showing young primary plasmodia (arrows) in root hairs. Magnification:a-c x1000.

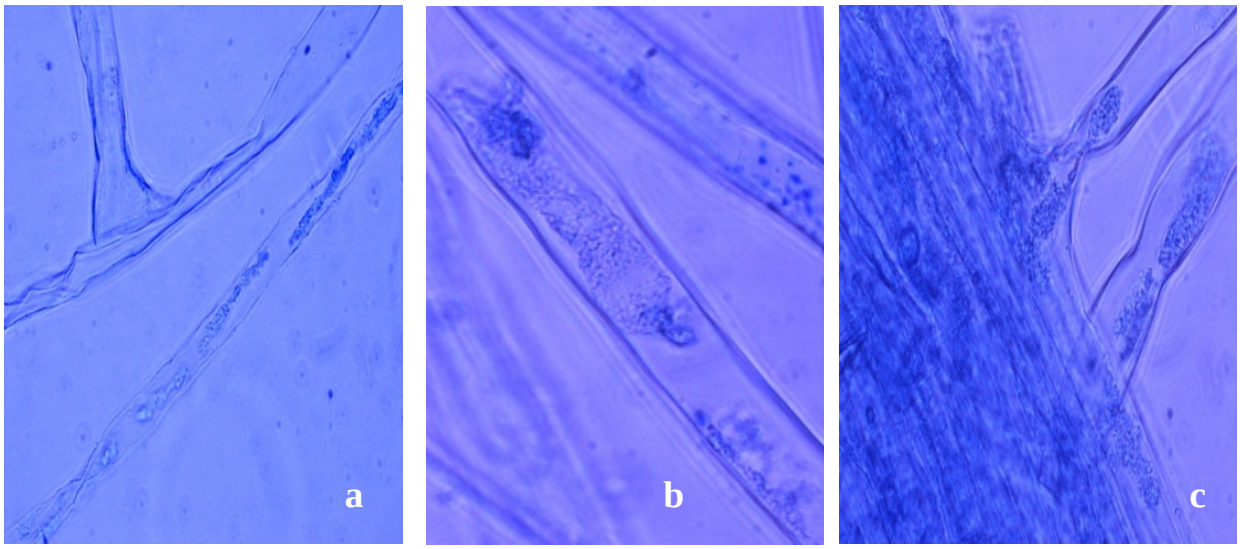

Figure 6.40: Stages in infection of resistant cabbage by population No. 4 at 2-4 dai, showing large, differentiating plasmodia (left+centre) and immature zoosporangia (right) in root hairs. Magnification: a x600, b-c x1000.

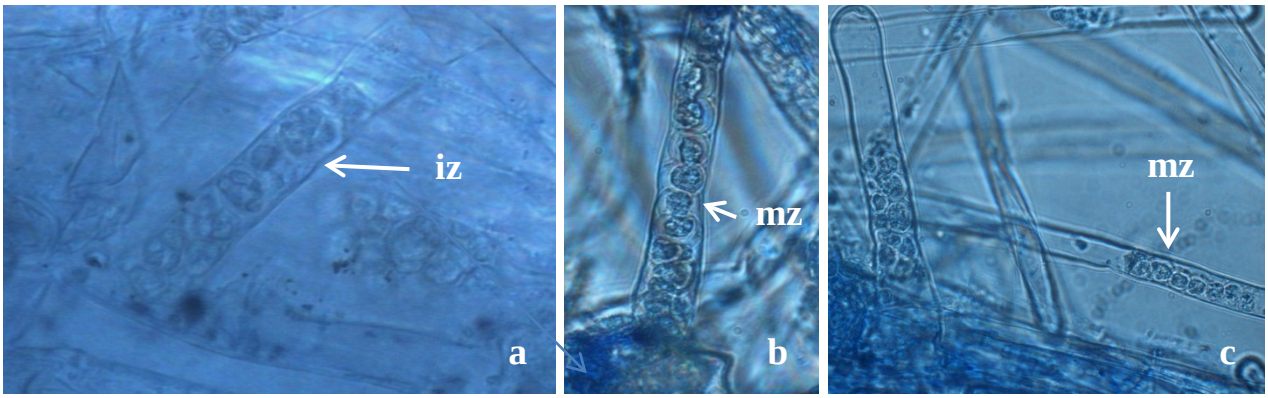

Figure 6.41: Stages in infection of resistant cabbage by population No. 4 at 10-14 dai, showing immature (iz) and mature, fully differentiated (mz) zoosporangia in root hairs. Magnification: a x1000, b-c x600.

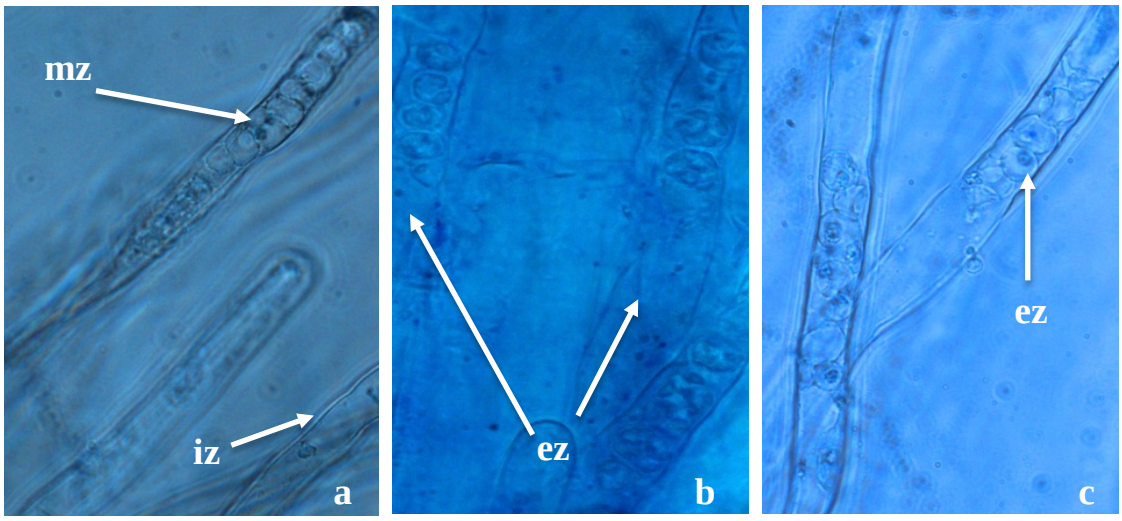

Figure 6.42: Stages in infection of resistant cabbage by population No. 4 at 15-17 dai, showing immature (iz), mature (mz) and empty (ez) zoosporangia in root hairs. Magnification: a-c x1000.

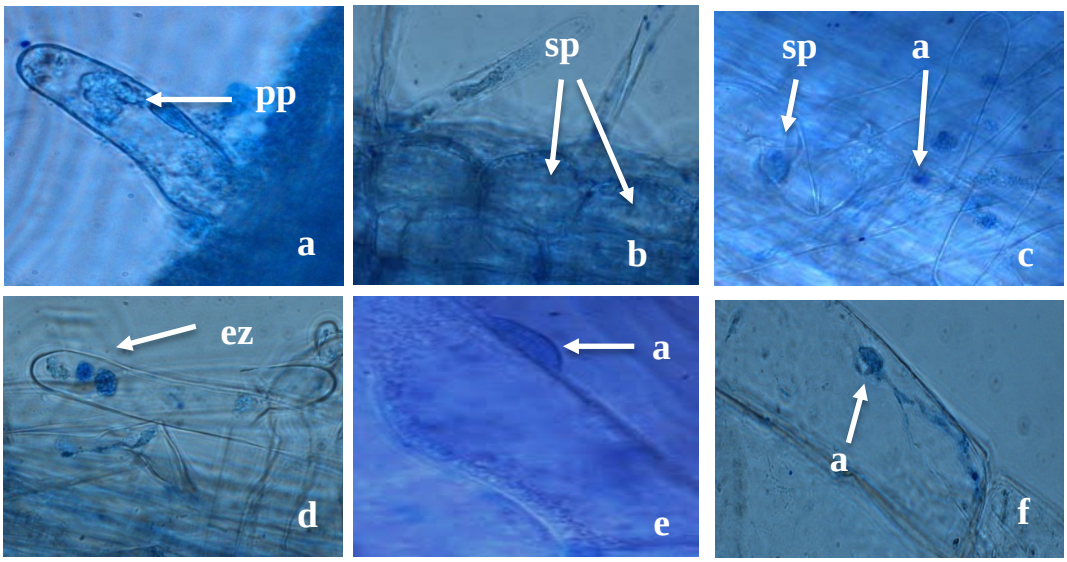

Figure 6.43: Stages in infection of resistant cabbage by population No. 4 at 18-30 dai, showing primary plasmodia (pp), empty zoosporangia (ez), secondary plasmodia (sp) and amoeboid (a) forms, and host cell nuclei (n). Magnification: a-d x1000, e-f x1600.

6.3.1.3.3 Differences in events with plant and pathogen types

Infection in both types of plant with both types of pathogen comprised the same six stages (**Table 6.6**). No differences were observed in the appearance of any of these stages between plant or pathogen types or their combinations. The timings of the various stages constituted wide ranges and overlapped to the extent that there was no consistent difference in timing between plant types (susceptible or resistant) or between pathotypes (lowly or highly virulent) (**Table 6.6**). The highly virulent population No. 4 perhaps produced primary zoospores that encysted and produced primary plasmodia a day or two earlier than the mildly virulent population No. 1, but it is difficult to be certain and earlier developing plasmodia may have been missed in the latter. The overall conclusion was that events, their timings and their appearances were the same regardless of plant resistance and pathogen virulence.

Table 6.6: Timing (dai) of events in infection of susceptible and resistant cabbage by *Plasmodiophora brassicae* pathotypes.

Stages observed in root hairs	**Population**			
	Mildly virulent population No. 1		**Highly virulent population No. 4**	
	Susceptible	**Resistant**	**Susceptible**	**Resistant**
Primary zoospore encysted on root hairs	1-4	2-3	1	1
Young primary plasmodia in root hairs	5-8	4-6	2-7	2-4
Large differentiating primary plasmodia and immature zoosporangia	9-12	7-12	8	5-9
Fully differentiated zoosporangia.	13-14	13-16	9-16	10-14
Zoospore release from root hairs evidenced by empty zoosporangia	15-17	17-18	17-30	18-30
Secondary plasmodia and amoeboid forms	17-30	19-30	9-30	18-30

6.3.1.4 Fixed and stained roots

6.3.1.4.1 Low virulence pathotype (population No. 1)

Both susceptible and resistant plants showed microscopic symptoms of infection. Susceptible plants showed each symptom earlier than resistant plants and the progression of the pathogen through the life cycle stages (plasmodia to resting spores) was more complete in susceptible than resistant plants.

6.3.1.4.1.1 Susceptible host plants

For the first 10 dai, the roots were healthy and cells retained their normal shape, size and nuclear appearance (**Fig. 6.44 a, b, c**). The first visible response of the roots to infection was at 8-10 dai in the form of the increased size of the nucleus and its fusiform shape (**Fig. 6.44 d, e**) although the cortex, cambium and vascular bundles remained intact (**Fig. 6.44 f**).

Galls were not visible in inoculated susceptible cabbage plants until 11 dai (**Fig. 6.44**) and were absent in inoculated resistant plants and uninoculated control plants. Only plasmodia were visible in roots at 11 dai.

In galls, larger infected and smaller uninfected cells were present, and both resting spores and plasmodia were observed at different stages of development (**Fig, 6.45**). Damage to the inner cortex was characterized by degradation of the cell walls, including the secondary thickened walls of the xylem. At 11-15 dai, early stages of infection began appearing in the endodermis and cortex as young and later maturing plasmodia (**Fig. 6.45 a, b and c**). Some of the young plasmodia appeared to have finger-like processes (Fig. 6.45a) but it was difficult to be sure from the magnification possible. From 16 dai, later stages of infection were observed (mature plasmodia and masses of resting spores) in infected cells (**Fig. 6.45 d-f**). This was accompanied by increased cell size (hypertrophy), disruption of the cell wall, and the presence of vesicles or inclusion bodies inside the cell. Evidence of recent cell division was present as groups of similar-sized small cells between groups of infected cells (**Fig. 6.45 d**), suggesting hyperplasia.

At 30 dai, *P. brassicae* had invaded 63% of cells (60% contained resting spores and 2.2% contained plasmodia) (**Table 6.5; Appendix 6, Table A6.5 a-c**).

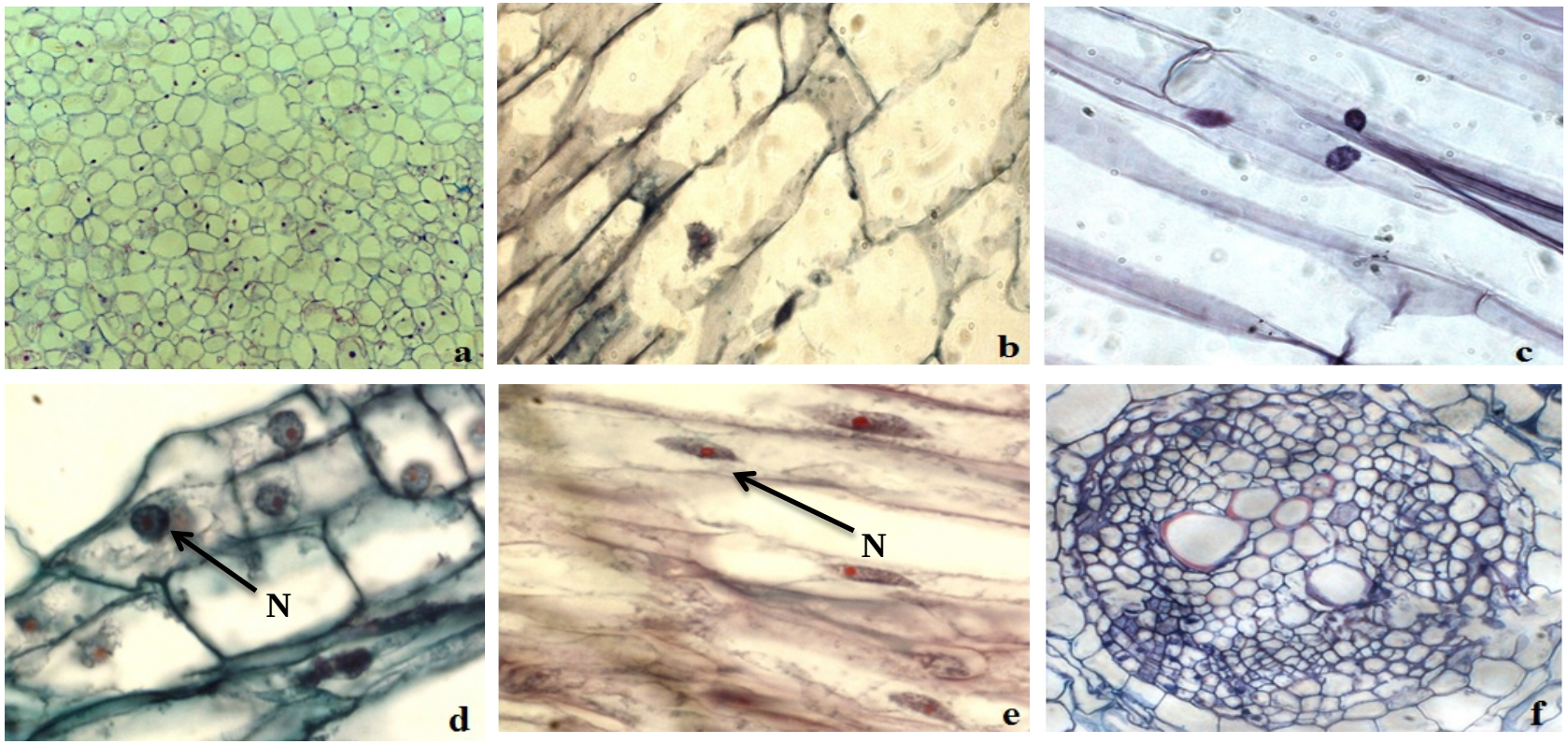

Figure 6.44: Section through roots of susceptible cabbage at 1-10 dai, showing that most cells look uninfected and healthy [clear cytoplasm and prominent nuclei (N)].(a) 1 dai, (b) 3 dai, (c) 4 dai, (d) 6 dai, (e) 8 dai, (f) 10 dai. Magnification: (a) x400, (b)-(f) x1000.

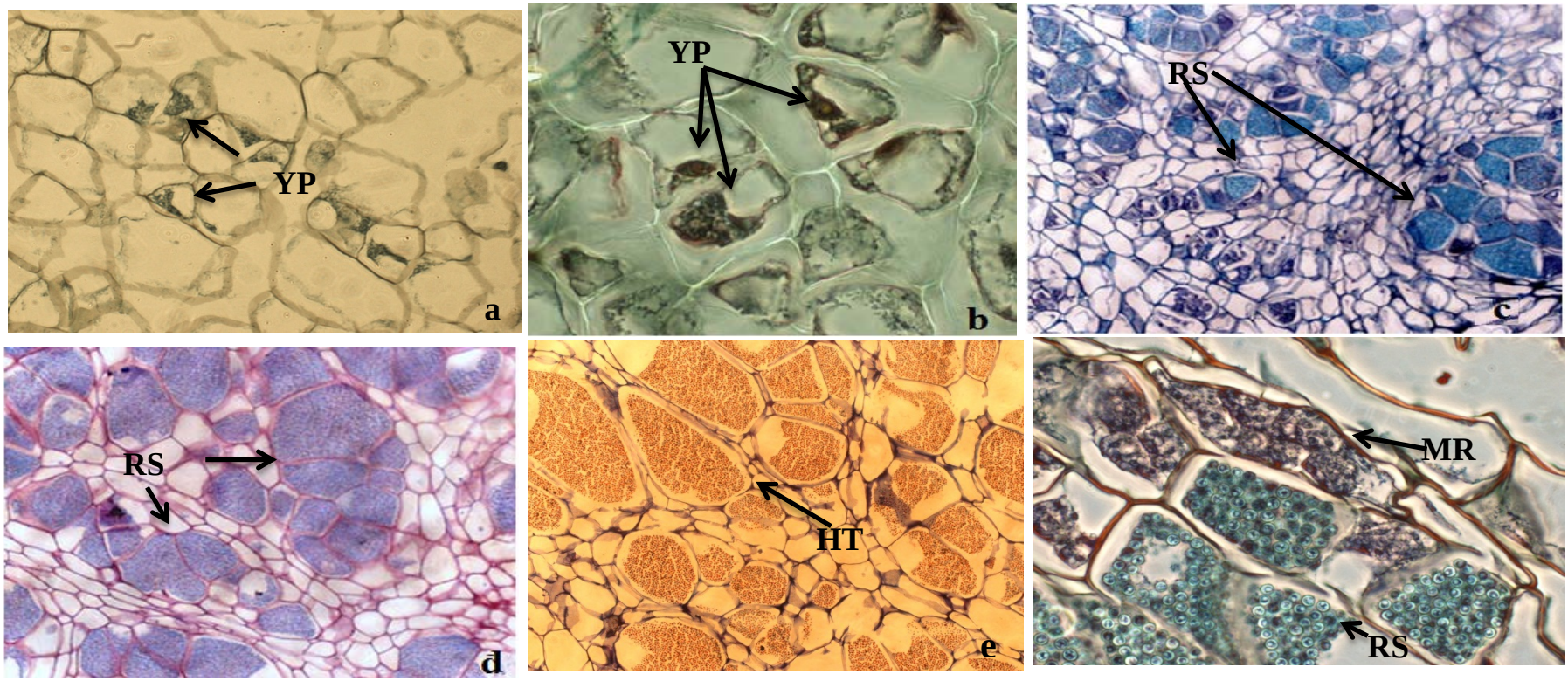

Figure 6.45: Sections through galls of susceptible cabbage at 11-28 dai with population No.1, showing stages in infection Young plasmodium (YP), mature plasmodium (MP), immature resting spores (IRS), mature resting spores (RS). Increased cell size (hypertrophy- HT). Magnification: a x600, c,d,e x1000; b-f x1600.

6.3.1.4.1.2 Resistant host plants

There was no sign of infection at 1-10 dai in resistant plants (**Fig. 6.17 a-c,f**). Uninoculated plants showed only healthy uninfected cells at all times.

The first evidence of cortical infection occurred at 12 dai, even though the cells were still healthy and the nuclei were ovoid and prominent (**Fig. 6.46 a-c**). The first sign of infection was the almond-shaped nucleus (**Fig.6.46 d-e**), even before any appearance of plasmodia (**Fig**. **6.47 a-c**).

Plasmodial stages occurred from 13 dai (**Fig. 6.47 a-c**) and in later stages many multinucleate plasmodia with different sizes and shapes were observed (**Fig. 6.47 c-d**). Some of the young plasmodia appeared to have finger-like processes (**Fig. 6.47 a,f**). The infected cells showed hypertrophy and cell wall rupture in the host plant (Fig. 6.47a,d). Mature plasmodia were in the outer cortex layer near the periderm (**Fig.6.47 b-c**), while young plasmodia were mainly in small cells next to the stele (**Fig. 6.47 c**). Resting spores were also present from 15 dai (**Fig. 6.47 f**). Some of the plasmodia at later stages showed that the contents had rounded up and these may represent early stages in resting spore formation (**Fig. 6.47 e-f**). Cell inclusions were also present at these later stages (**Fig. 6.47 f**).

At 30 dai, 1% of the cells contained resting spores, 59% contained plasmodia and 40% were uninfected (**Table 6.7; Appendix 6, Table A6.7 a-c**). The proportions of uninfected cells, and of infected cells with resting spores, were twice those of susceptible plants.

The proportions in the different types of cell were significantly different between susceptible and resistant host plants (χ^2 = 7065, DF = 2, P-Value = <0.001).

Table 6.7: Numbers and proportions of types of cells at 28 dai in galls of cabbage (*Brassica oleracea)* inoculated with lowly virulent population No. 1 of *P. brassicae.*

Parameter	Susceptible hosts			Resistant hosts		
	Resting spores	**Plasmodium**	**Intact cells**	**Resting spores**	**Plasmodium**	**Intact cells**
Total cells	3868	142	2406	89	3854	2625
Percentage	60%	2%	38%	1%	59%	40%

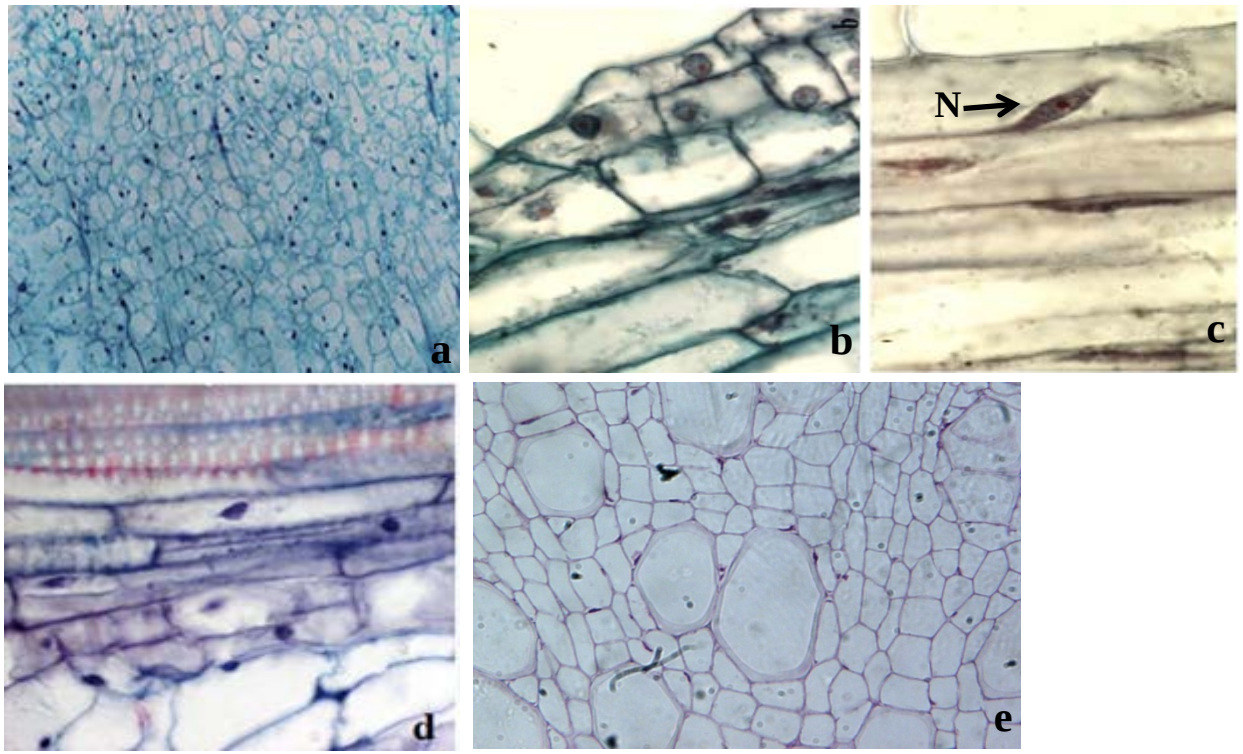

Figure 6.46: Sections through roots of resistant cabbage at 1-12 dai with population No. 1, showing healthy tissue and early stages of infection. (a) 1dai, (b) 3 dai, (c) 5 dai, (d) 8 dai, (e) 12 dai . Nucleus (N). Magnification: a,e x600, b,c,d x1000.

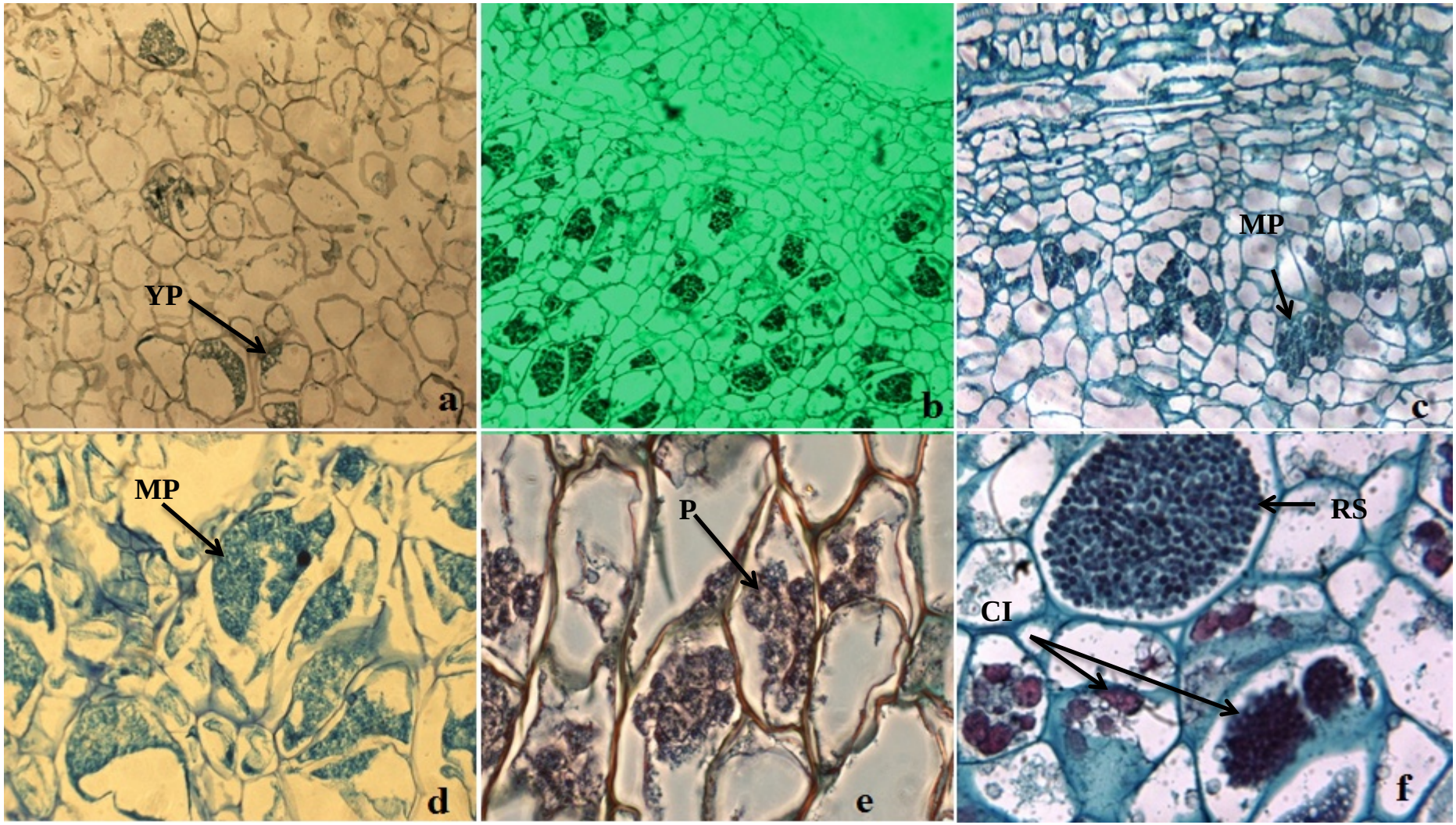

Figure 6.47: Sections through roots of resistant cabbage at 15-28 dai with population No.1, showing the presence of infection. Young plasmodium (YP), plasmodium (P), mature plasmodium (MP), mature resting spores (RS), cell inclusions (CI). Magnification: a-d x1000, e-f x 1600.

6.3.1.4.2 High virulence pathotype (population No. 4)

Plasmodiophora brassicae infected both susceptible and resistant hosts. Symptoms of cortical invasion included cell wall breaks, cell wall thickening and enlarged and/or disorganized cells with fusiform host nuclei. However, damage to the inner cortex was observed only in susceptible hosts and was characterized by degradation of the cell walls and secondary thickening of the xylem.

6.3.1.4.2.1 Susceptible host plants

For the first 7 days there were no microscopic symptoms of cortical invasion. Cells were healthy and free from infection in the epidermis, cortex and vascular tissue (**Fig. 6.48 a-f**). Resting spores or their empty shells were present on the surface of the epidermal cell wall (**Fig. 6.48 d**). The nuclei were prominent and later changed to a fusiform shape (**Fig.6.48 f**). Zoosporangia were observed in root hairs at 7 dai (**Fig. 6.49 a**).

By 11 dai plasmodia were observed (**Fig. 6.49 b-d**). Resting spores appeared from 2 days later (**Fig. 6.50 a**). Infection was associated with cell wall breakage and damage to the inner cortex (**Fig. 6.51 a-b**). The proportion of infected cells containing resting spores increased at 18-28 dai (Fig. 51a-d). By 28 dai 87% of the host tissue contained resting spores, 7% contained plasmodia and only 6% of cells were uninfected (**Table 6.6; Appendix 6, Table A6 a-c**).

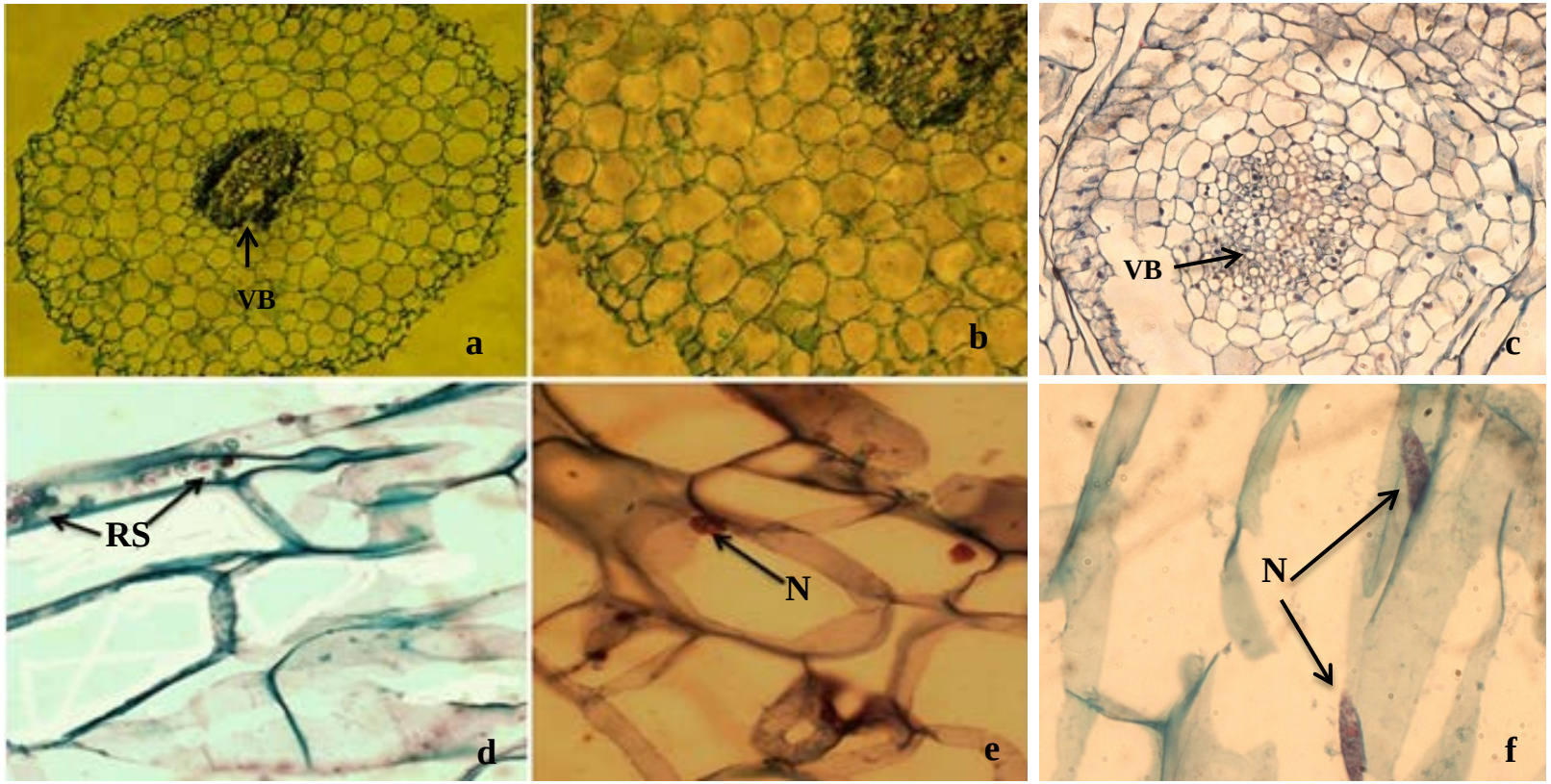

Figure 6.48: Sections through roots of susceptible cabbage at 1-11 (dai) with population No.4, showing cells healthy and uninfected. Nucleus (N), resting spores (R), vascular tissue (VB). dai, (b) 3 dai, (c) 5 dai, (d) 5 dai, (e) 8 dai, (f) 11 dai. Nucleus (N), resting spores (R), vascular tissue (VB). Magnification: a-c x600, d-f x1000.

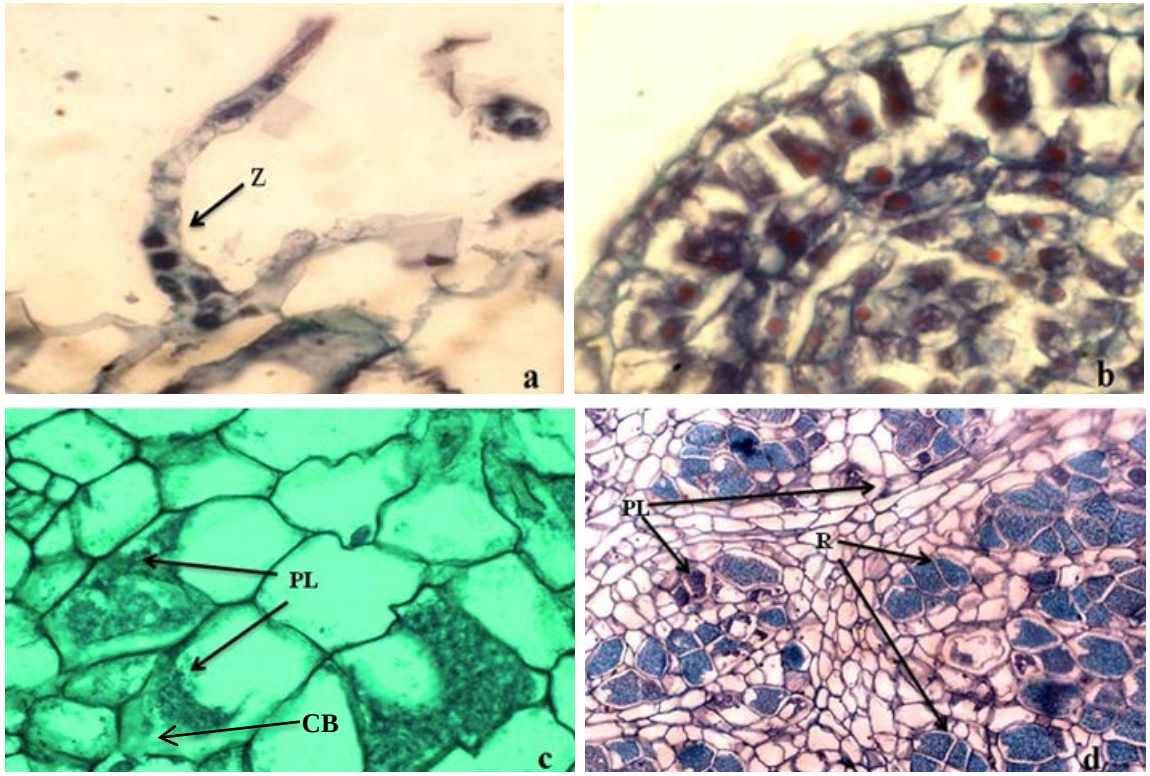

Figure 6.49: Sections through roots of susceptible cabbage at 12-18 dai with population No.4, showing many cells infected. (a) 11 dai, (b) 12 dai, (c) 14 dai, (d) 16 dai. Cell wall breakage (CB), plasmodia (PL), resting spores (R), zoosporangia (Z), Nucleus (N). Magnification: a-d x1000.

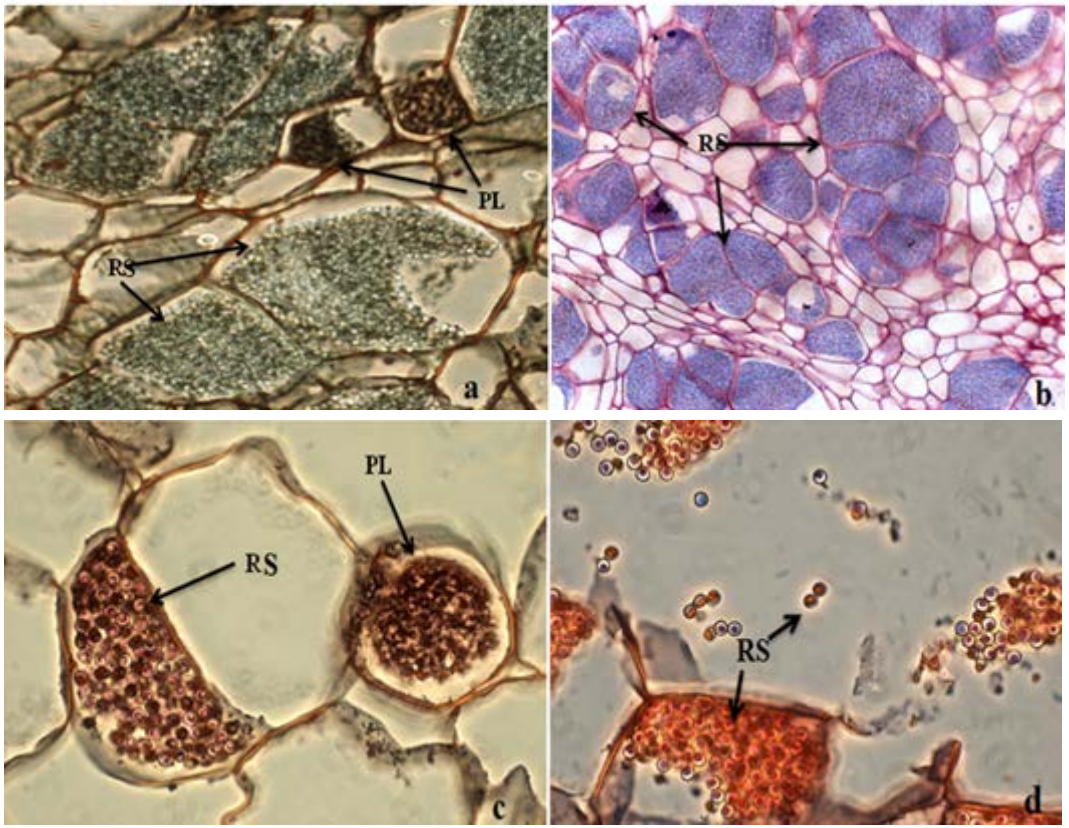

Figure 6.50: Sections through roots of susceptible cabbage at 18-28 dai with population No.4, showing most infected with resting spores. (a) 18 dai, (b) 20 dai, (c) 16 dai, (d) 28 dai. Plasmodia (PL), resting spores (RS). Magnification: a-d x1000.

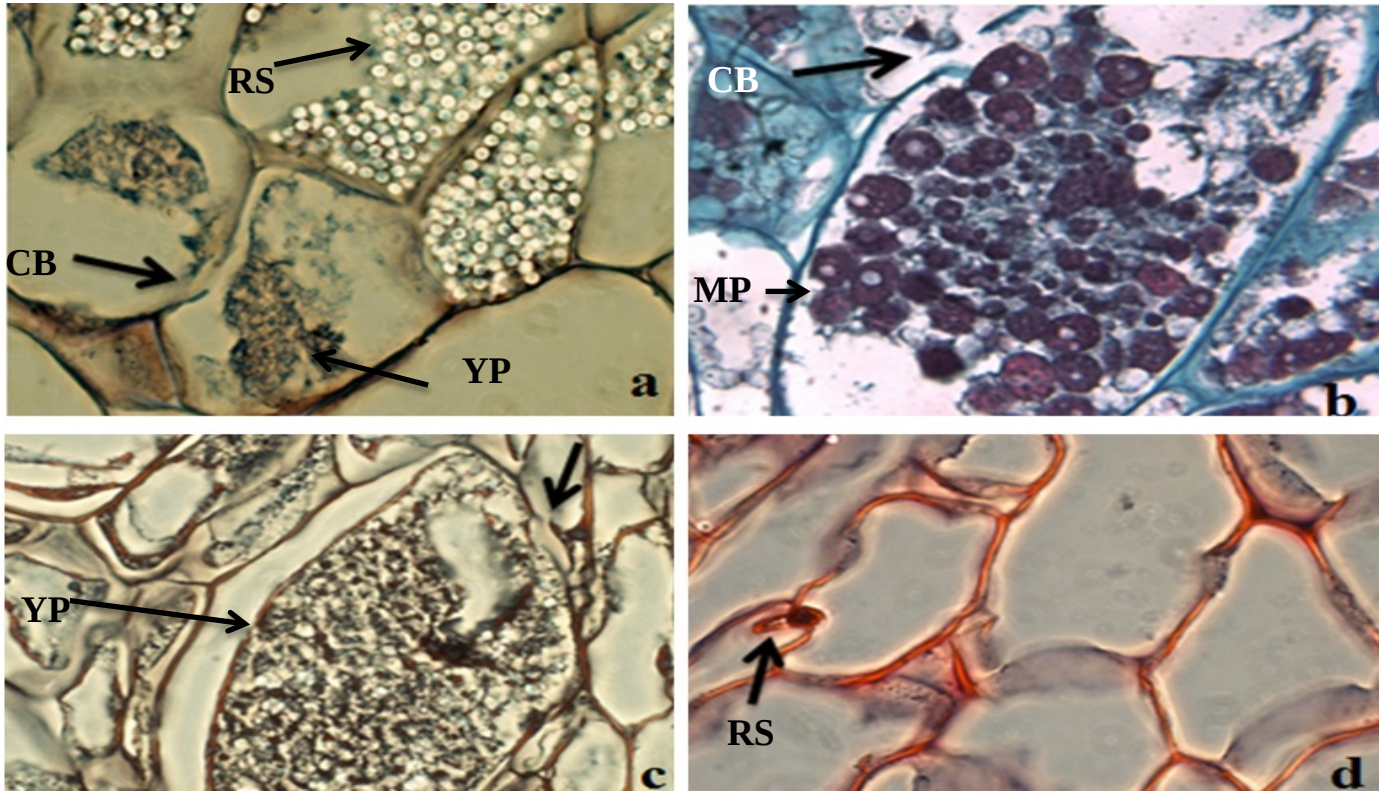

Figure 6.51: Sections through roots of susceptible cabbage with population No. 4 at 20 dai, showing maturation of plasmodia and resting spores Young plasmodia (YP), mature plasmodia (MP), resting spores (RS), Cell wall breakage (CB). Magnification x=1000.

6.3.1.4.2.2 Resistant host plants

No infection was seen microscopically until 10 dai, although resting spores were observed on the surface of the root (**Figs 6.52 a-b, 6.52 d**) and young plasmodia (**Fig. 6.52 c-d**).

As in susceptible plants, most cortical cells in resistant plants became infected during the next 18 days. Infected cells were enlarged compared with uninfected cells and contained plasmodia (**Figs 6.52 d, 6.53 a-e**). Cell wall breakages were observed (Figs **6.52 b, 53 e**) but not in the inner cortex. Within infected cells, some plasmodia matured and developed into resting spores (**Figs 6.52 d, 6.53 c**) but most did not mature into resting spores at any stage of infection, although there was evidence of their differentiation inside mature plasmodia (**Fig. 6.53 b,d**).

By 28 dai, 92% of host cells were infected (84% containing plasmodia) (**Table 6.8; Appendix 6, Table A6.8 a-c**). This contrasted markedly with susceptible plants, where most infected cells contained resting spores.

The proportions in the different types of cell were significantly different between susceptible and resistant host plants (χ^2 = 11972, DF = 2, P-Value <0.001).

Table 6.8: Numbers and proportions of types of cells at 28 dai in galls of cabbage (*Brassica oleracea)* inoculated with highly virulent population No. 4 of *P. brassicae.*

Parameter	**Susceptible host**			**Resistant host**		
	Resting spores	**Plasmodium**	**Intact cells**	**Resting spores**	**Plasmodium**	**Intact cells**
Total cells	7734	625	525	694	7752	718
Percentage	87%	7%	6%	8%	84%	8%

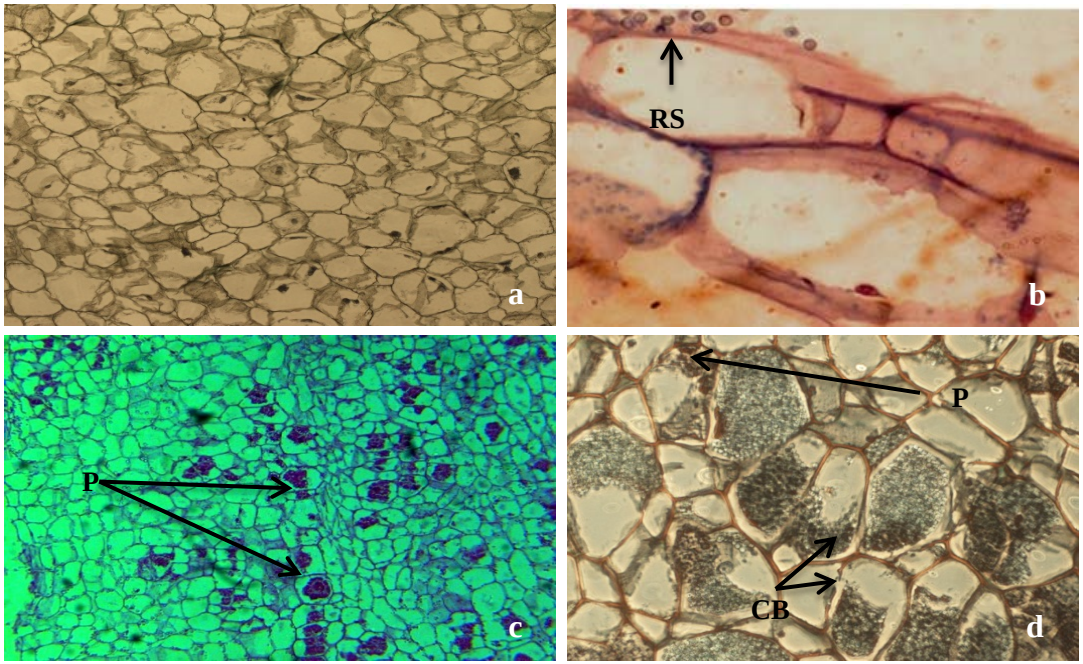

Figure 6.52: Sections through roots of resistant cabbage at 1-15 dai with population No. 4, showing increasing numbers of cells infected. (a) 1 dai, (b) 3 dai, (c) 11 dai, (d) 13 dai. Plasmodia (P), resting spores (RS). Magnification: a,c and d x6000, b x1000.

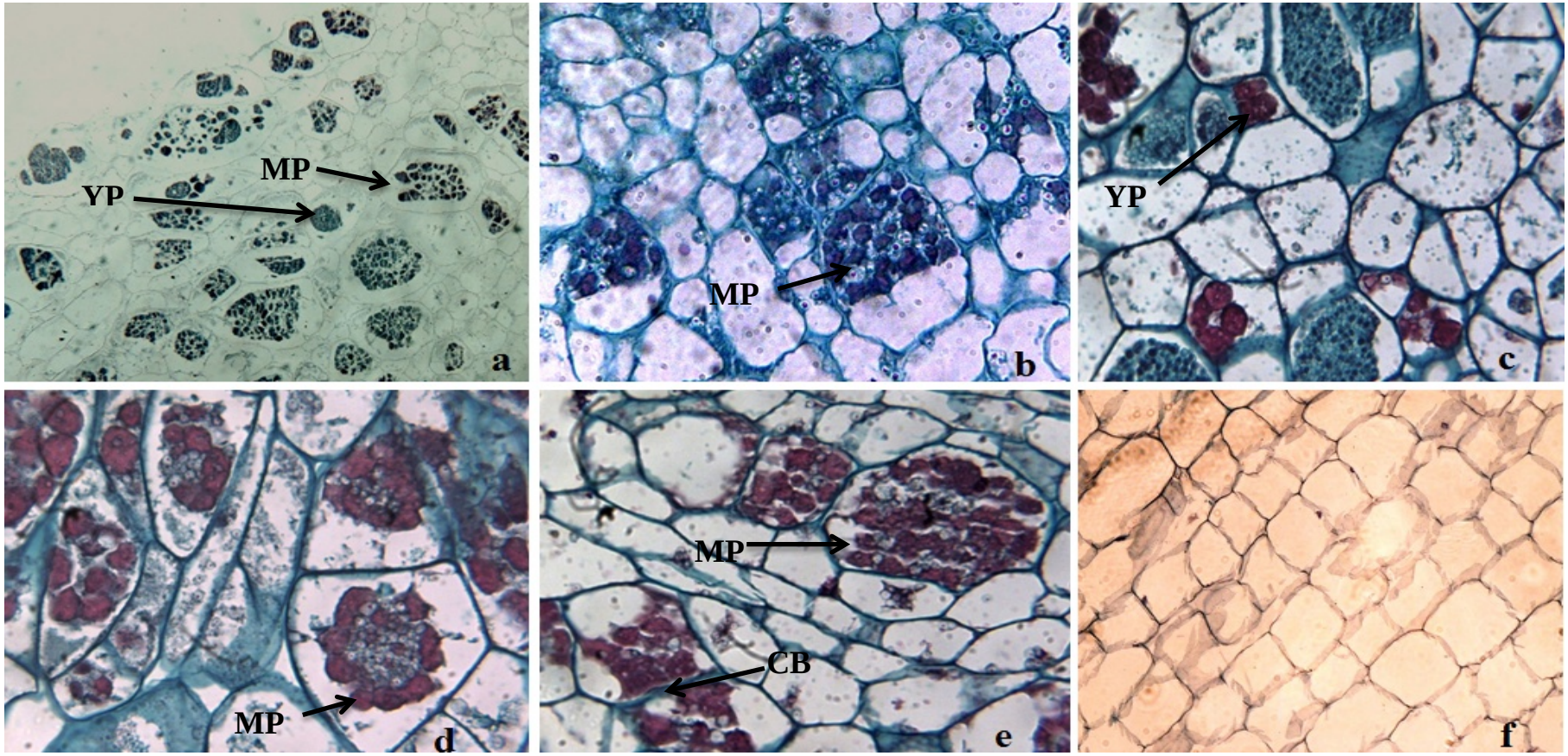

Figure 6.53: Sections through roots of resistant cabbage at 16-28 dai with population No.4, showing maturation of plasmodia. (a) 15 dai, (b) 18 dai, (c) 19 dai, (d) 23 dai, (e)28 dai, (f) intact cells . Young plasmodia (YP), mature plasmodia (MP), cell wall break (CB). Magnification: a-f x600.

6.3.2 Chinese cabbage (*B. rapa* var. *chinensis*)

6.3.2.1 Macroscopic appearance

For both susceptible and partially resistant host plants, galls were formed on roots (since no true resistance to *P. brassicae* is known in Chinese cabbage) and sections showed that galls contained both infected and uninfected cells. Cells infected with *P. brassicae* were larger than uninfected cells, indicating hypertrophy.

6.3.3.2 Fixed and sectioned roots

Resting spores and plasmodia were observed in roots along with uninfected cells (**Fig. 6.54**), but the proportions of each were different (**Table 6.9; Appendix 6, Table A6.9 a-c**). Susceptible plant roots had greater proportions of infected cells, particularly those with resting spores, than did partially resistant plant roots. In susceptible plants the ratio of cells with resting spores:plasmodia:uninfected was 72:7:21 (**Fig. 6.54 a,b**), whereas in resistant plants the ratio was 2:59:39 (**Fig. 6.54 c,d**). The proportions in the different types of cell were significantly different between susceptible and partially resistant host plants (χ^2 = 5496, DF = 2, P-Value <0.001). Susceptible plants had greater proportions of infected cells and of resting spores than partially resistant plants.

Table 6.9: Numbers and proportions of types of cells at 56 dai in galls of Chinese cabbage (*Brassica rapa* var. *chinensis* cv. Granaat) inoculated with *P. brassicae* strain S.

Parameter	Susceptible host			Partially resistant host		
	Resting spores	Plasmodium	Intact cells	Resting spores	Plasmodium	Intact cells
Total cells	4476	444	1329	93	2301	2104
Percentage	72%	7%	21%	2%	59%	39%

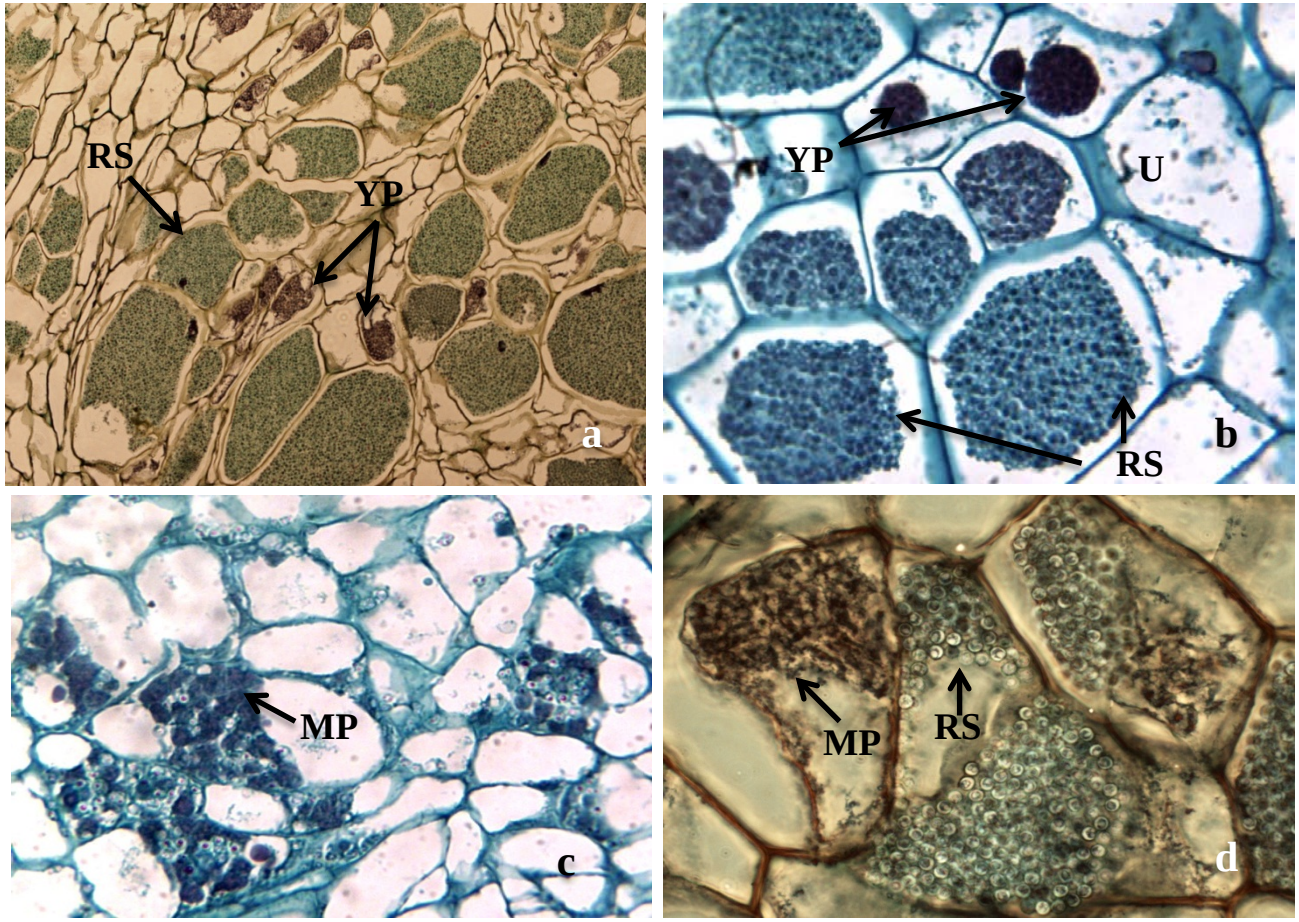

Figure 6.54: Gall sections of susceptible (a, b) and resistant Chinese cabbage. (c, d) showing different stages of *Plasmodiophora brassicae* in cells: resting spores (RS), young plasmodium (YP), mature plasmodium (MP), uninfected cells (U). Magnification: a-c x600, b-d x1000.

6.3.3 Data analysis

The relative distributions of intact (uninfected) cells, infected cells with resting spores and infected cells with plasmodia suggested significant differences between the two types of cabbage and between the two types of inoculum (**Figs. 6.55- 6.56**),

Data and analyses of individual combinations of plants and pathogens have been presented previously (**Sections 6.3.1.4, 6.3.2; Tables 6.7-6.9**) and showed that there were significant differences in proportions of cells within each resistance plant type for any one inoculum. Further analysis for cabbage showed that plant resistance type made a significant difference when both pathogen populations were considered (**Table 6.10**) (χ^2 = 19261, DF = 5, P-Value <0.001 for differences between host types). Pathogen population type also made a significant difference when both plant resistance types were considered (**Table 6.11**) (χ^2 = 4969, DF = 5, P-Value <0.001 for differences between pathogen types).

Table 6.10: Numbers of types of cells at 28 dai in galls of cabbage (*Brassica oleracea)* in plants differing in resistance and inoculated with either pathogen population.

Cabbage	**Population No. 1**			**Population No. 4**		
Parameter	**Resting spores**	**Plasmodium**	**Intact cells**	**Resting spores**	**Plasmodium**	**Intact cells**
Susceptible host	3868	142	2406	7734	625	525
Resistant host	89	3854	2625	694	7752	718

Table 6.11: Numbers of types of cells at 28 dai in galls of cabbage (*Brassica oleracea)* inoculated with low (No. 1) or high (No. 4) virulence populations of *P. brassicae.*

Cabbage	**Susceptible hosts**			**Resistant hosts**		
Parameter	**Resting spores**	**Plasmodium**	**Intact cells**	**Resting spores**	**Plasmodium**	**Intact cells**
Popn No. 1	3868	142	2406	89	3854	2625
PopnNo. 4	7734	625	525	694	718	7752

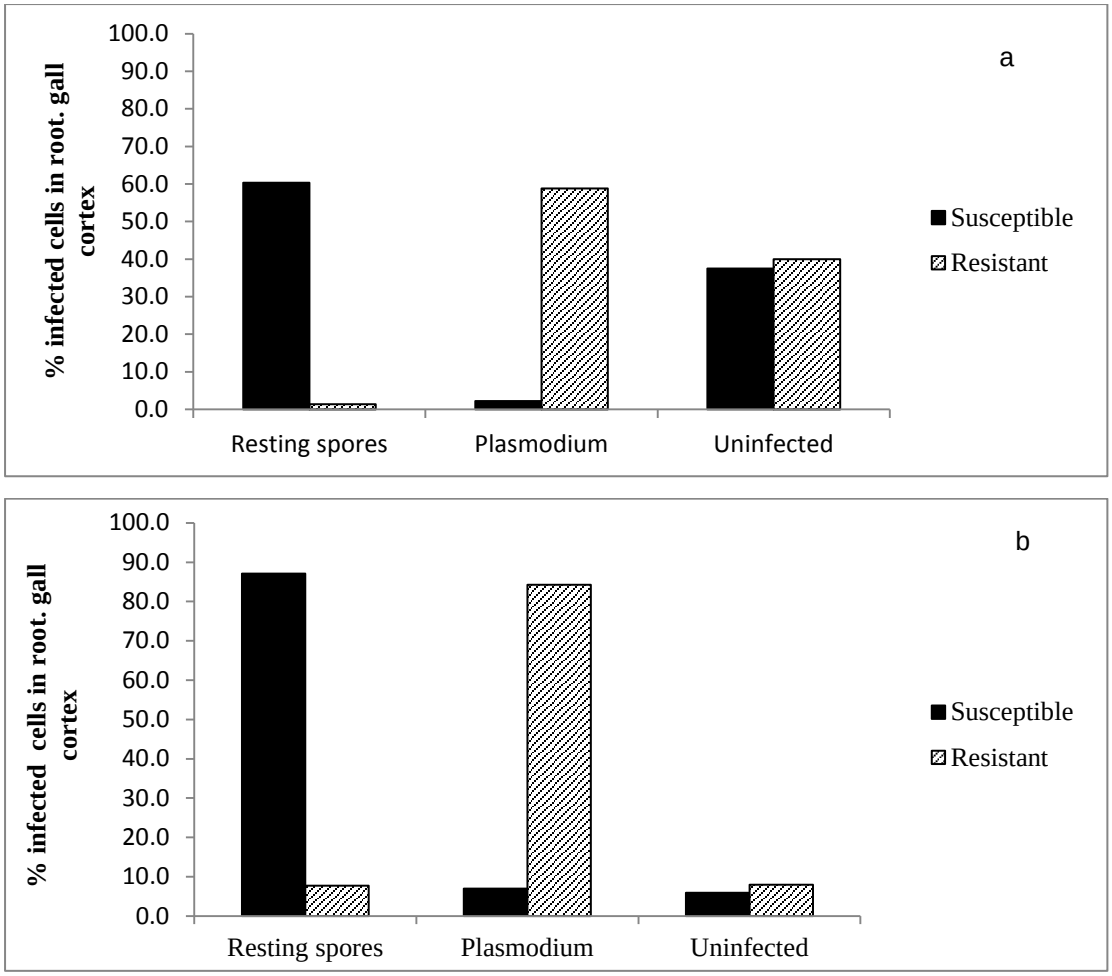

Figure 6.55: Variation in cell contents at 28 dai between cabbage lines Syngenta Stock ID. CA0826 (susceptible) and Maxfield F1 CRR (resistant - no visible gall development) inoculated with (a) low virulence population No. 1 and (b) high virulence population No. 4.

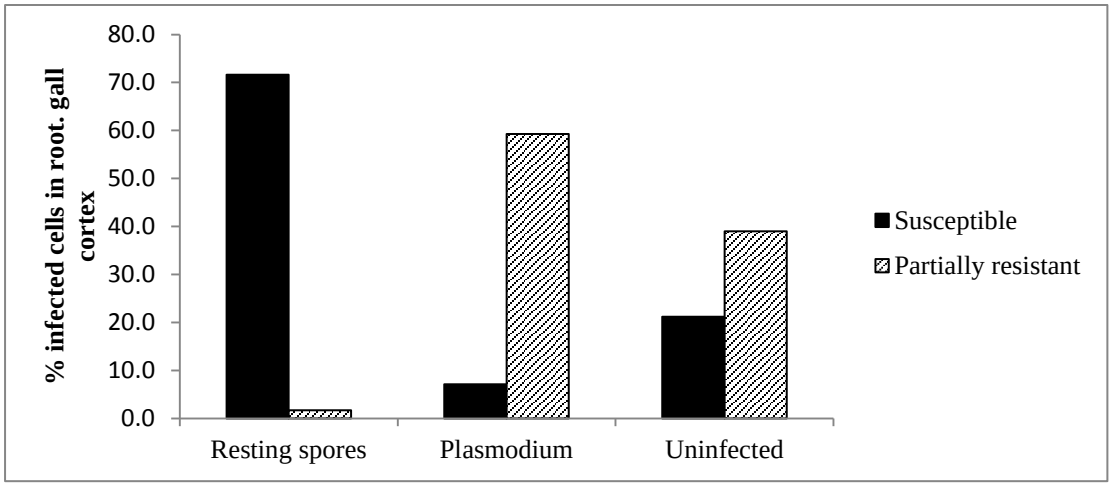

Figure 6.56: Variation in cell contents at 56 dai with variation in resistance of Chinese cabbage 'Granaat' (susceptible) and 'Tahono' (partially resistant) inoculated with the highly aggressive strain S (Launching Place, Victoria).

In cabbage, the major difference between plant resistance types was that the infected cells in susceptible plants contained mostly resting spores, whereas those in the resistant plants contained mostly plasmodia (**Fig. 6.55a**). This occurred with both pathogen types. The data do not reliably compare the proportions of cells infected because not all sections had infection and so the apparent increase in the percentage of cortical cells infected from about 60% with the low virulence population No. 1 to over 80% with the high virulence population No. 4 underestimates the effect of virulence on reducing the number of cells infected (**Fig. 6.55b**). This is the case with both plant types. Even though the resistant plant roots had pathogen in all stages of its life cycle, they did not develop galls. Inspection of the total cell counts in microscope fields of view showed comparable numbers of cells counted and so it is likely that that the degree of hypertrophy was similar.

In Chinese cabbage with the highly aggressive population S, a combination of effects was observed: partially resistant plants had greater proportions of infected cells in the cortex and these mostly contained plasmodia, whereas susceptible plants contained mostly resting spores (**Fig. 6.56**).

Although these were two different plant species with different amounts of resistance, there were some trends in common:

- for resistant plants compared with susceptible plants, there was no difference in the proportion of cells infected, but fewer infected cells developed resting spores.
- susceptible plants developed more resting spores than plasmodia in infected cells, whereas resistant plants developed more plasmodia than resting spores.

6.4 Discussion

This study of the morphological phases of infection by *P. brassicae* has shown that most phases seen by others were also seen in these studies of susceptible and resistant cabbage (*B. oleracea)* and susceptible and partially resistant Chinese cabbage (*B. rapa* var. *chinensis).* The features seen were similar to previous reports on differences between susceptible and resistant *Brassica* species, *R. sativus* and *A. thaliana* (reviewed by Kageyama and Asano 2009). Overall, these suggested that primary root hair infection was not limiting to host root infection and the ability to limit *P. brassicae* secondary infection in the cortex and delay or prevent the completion of its life cycle (by forming resting spores) was much more important in reducing the effects of infection. This agrees with previous reports on other species infected by *P. brassicae.*

1. little or no qualitative difference with plant or pathogen type was observed in the morphology and timing of primary root hair infection but there were quantitative differences in secondary infection of the internal tissues of the root
2. there was no evidence for vertical resistance even in cultivars that did not develop macroscopically visible galls.

The approach of using pathogen x resistance type rather than varying only one enabled the clarification of criteria attributable to each organism. Major conclusions from the study of these infections in these two *Brassica* species are listed below and discussed in more detail later:

1. virulence in the pathogen populations was associated with greater infection of host cortical cells but not with greater proportions of cells forming resting spores
2. resistance in plants was associated with lesser proportions of resting spore formation in infected cells and with reduced infection of host cortical cells in Chinese cabbage.

6.4.1 Infection processes

6.4.1.1 Primary infection in root hairs

There is little evidence to suggest any difference in primary infection with plant or pathogen type. Only two qualitative differences were noted, though a more quantitative study might find more evidence.

The greater persistence of resting spores on the root hair and epidermal surfaces by SEM with the high virulence population No. 4 than the low virulence population No. 1 has several

possible interpretations. It may suggest greater longevity in intact resting spores, giving greater opportunities for infection, but it would seem advantageous for the pathogen for as many resting spores to germinate as quickly as possible, as high spore doses increase infection (Naiki et al. 1987; Kobelt et al. 2000). Resting spores may also be chemically attached to roots, though there is no evidence for this in the literature, unlike in rhizobia, where the bacteria are attracted specifically to the root hairs of host plants and become attached by lectin bridges followed by encasement in the cell wall by newly produced cellulose microfibrils (Bohlool and Schmidt 1976). Alternatively, the early disappearance of resting spores in the low virulence population may be the result of their greater germination, though this seems unlikely. Only a detailed study that includes recovery of primary zoospores and empty resting spores could resolve this.

In root hairs, plasmodia from encysted zoosporangia became multinucleate, developed into zoosporangia and presumably released secondary zoospores, as empty zoosporangia were observed, as described previously by others (Aist and Williams 1971; Asano et al. 1999; Ingram and Tommerup 1972; Kroll et al. 1983; Mithen and Magrath 1992; Naiki et al. 1984; Tommerup and Ingram 1971; Woronin 1878).

Any differences in the timing of primary root hair infection (light microscopy) between plant or pathogen types were minimal, as ranges overlapped, with the possible exception of earlier zoospore encystment with the high virulence population No. 4 than with the low virulence population No. 1. The evidence for 1-2 day earlier encystment is not strong, as small numbers of early encysted zoospores could easily have been missed during examination, and so differences in timings of this magnitude are not reliable without further quantitative analysis. If earlier timing does exist with population No. 4 than population No. 1, the reasons may be differential mortality or activity of the primary zoospores (Ikegami, 1985; Murakami et al., 2001). Further investigations should use a hydroponic system similar to that here, as it has no barriers to pathogen access to host plants and infection depends only on each population's ability to penetrate the host. A quantitative rather than the qualitative approach as used here would be needed to demonstrate differences.

The lack of difference in the timing of these primary stages in root hairs is consistent with previous studies of clubroot infection (Ayers 1944; Dekhuijzen 1979; Donald et al. (2008); Kroll et al. 1983; Macfarlane 1955; Naiki et al. 1987).

Differences at this primary infection stage would not be expected, however, as the root hair stage is generally agreed not to be specific. Infections of root hairs by primary zoospores of *P. brassicae* occur even on non-host species (Macfarlane 1970) and Rashid et al. (2013) recently showed that root exudates from some non-hosts stimulated more resting spore germination than those from hosts. Also, Tanaka et al. (2006) found no correlation with final cortical infection in differentially resistant *B. rapa* ssp. *pekinensis*. This also fits with the observations of infected cortical cells even in plants without macroscopically visible galls (Donald et al. 2008; Kobelt et al. 2000; Osaki et al. 2008b), suggesting lack of vertical resistance (as defined by Van der Plank in 1963) (Agrios 2005) and necrosis of the infected cells and those immediately around them.

6.4.1.2 Secondary infection of the internal tissues

Infection events in the internal tissues of the root followed those described by other authors: small and large plasmodia were seen in hypertrophied infected cells, as were resting spores, along with cell wall breaks, disorganisation in cytoplasm and hyperplasia leading to disorganisation of the cortex and stele. These features are in common with those described previously (e.g. Dekhuijzen 1979; Donald et al. (2008); Gustafsson et al. 1986; Kobelt et al. 2000; Ludwig-Müller and Schuller, 2008; Mithen and Magrath 1992; Suwabe et al. 2003; Williams et al. 1967) and many others and summarised by Kageyama and Asano (2009). There was no obvious difference in frequency of cell wall breaks (unlike Donald et al. 2008), though quantitative study would be required to be certain. There was also no evidence of necrotic areas characteristic of hypersensitivity, as noted by some authors (Donald et al. 2008; Kroll et al. 1983; Tanaka et al. 2006), unlike others (Dekhuijzen 1979; Kobelt et al. 2000; Takahashi et al. 2006).

There was some evidence of an amoeboid stage in young plasmodia in both susceptible and resistant plants of both species, as noted by Donald et al. (2008) in *B. oleracea* (cauliflower) with the same resistance genes, but without TEM it was impossible to be sure. Material was fixed and embedded for TEM at each dai, but the dismantling of electron microscopic facilities on the Bundoora campus of RMIT University led to insufficient time to section and examine it. With TEM, it would be possible to be certain about features such as myxamoebae, secondary cell wall degradation in the xylem and amyloplast differentiation and location such as those noted by other authors (e.g. Donald et al. 2008; Mithen and Magrath 1992). It would also clarify the identity of the globose heavily stained structures in

older plasmodia in resistant plants, which have been interpreted as immature differentiating resting spores.

No conclusions about the presence and contents of amyloplasts or about the distribution and number of lipid droplets in the plasmodia were possible with the safranin-fast green stain used; different stains, such as periodic acid-Schiff or iodine for starch and safranin black for lipids would help to elucidate this problem, as would TEM of the material.

6.4.2 Virulence in the pathogen

In cabbage, the main differences in cortical infection between the two pathogen populations were the earlier timing and greater amount of cortical infection with both types of plant. Both of these observations can be attributed to the differential ability of each pathogen to invade the host plants at depth, as a reflection of its degree of virulence, and to the ability of the host plant to slow it, as a reflection of its degree of resistance.

In timing, the first sign of root swelling (gall formation) was 7 days earlier with the high virulence population No. 4 than with the low virulence population No.1, which suggests earlier and greater development of hypertrophy and/or hyperplasia is associated with greater virulence. In particular, gall formation was proposed to result from hyperplasia induced by invasion of the pericycle and vascular cambium (Kobelt et al. 2000; Malinowski et al. 2012) and 7 days may be the difference in time between the populations in reaching it, causing hyperplasia and causing the root to swell into a macroscopically visible gall. Greater proportions of cortical cells were infected by the high virulence population No. 4. The association of increased virulence level in the pathogen with increased cortical infection is similar to the results of others (Deora et al. 2013; Kobelt et al. 2000; Tanaka et al. (2006). The progression of 1-2-nucleate plasmodia to those with >9 nuclei seemed to be a critical stage in gall development (Tanaka et al. 2006) and increasing virulence gave greater proportions of infected cells in this category. This suggests that this is a vital stage in the development of the hyperplasia that characterises galls and is thought to be the result of cytokinin (Reddy and Williams 1970). Alternatively, the formation of resting spores may be a trigger for gall formation, as the chitin in their cell walls would be a potent signal to the host plant and liable to result in the production of chitinase and other pathogenesis-related enzymes (Cao et al. 2008; Kong Kaw Wa 2009).

Virulence in the SSI e3 was increased in a *jar1* mutant of *A. thaliana* in which the methyl jasmonate defence system was disrupted (Siemens et al. 2002). This suggests that greater

virulence increases the up-regulation of genes for both auxin and cytokinin production in gall tissue by down-regulating the jasmonate system at late developmental stages (multicellular plasmodia and resting spores), resulting in the rapid swelling of roots into galls (Jubault et al. 2008). However, this may not be true of all populations or isolates and ecotypes of *A. thaliana*, as Agarwal et al. (2011) found no effect of the *jar1* mutation on clubroot symptoms with an Australian field population of *P. brassicae*.

6.4.3 Resistance in the plant

In both species investigated, the association of increased resistance in both *Brassica* species with the predominance of plasmodial over resting spore stages in infected cortical cells suggests that the main mechanism of resistance in these *Brassica* species is to delay or prevent the completion of the life cycle of *P. brassicae* rather than to prevent cortical cell infection and is similar to observations by others (Osaki et al. 2008b; Kroll et al. 1983; Tanaka et al. 2006). Kroll et al. (1983) noted that resistant radish plants had uni- and bi-nucleate secondary plasmodia in similar numbers but that these never developed further in resistant plants and their development into more than nine-nucleate plasmodia was delayed by 12 days in partially resistant plants. This appears to be a critical stage in the development of hyperplasia and may correspond to about 12-16 dai in these plants when macroscopic galls developed on susceptible but not resistant cabbage roots, though some large plasmodia were visible in both types of plant. Kong Kaw Wa (2009) noted that the partially resistant 'Tahono' had reduced hyperplasia at 28 dai but that heavy hyperplasia developed later.

Partial resistance in these Chinese cabbage plants was also associated at 56 dai with less cortical cell infection. This is similar to reductions in the proportions of cells infected in resistant plants noted by others (Kobelt et al. 2000; Kroll et al. 1983; Morgner and Sacristan 1995; Tanaka et al. 2006). In cabbage, there was no accurate count of all infected and uninfected cells because many of the sections in the resistant plant roots had no infected cells and so only those sections with infected cells were counted, similar to Tanaka et al. (2006). In these sections, there seemed to be no difference in the numbers of cells for resistant and susceptible plants, suggesting that the average size of cells was the same and that the degree of hypertrophy was the same, though confirmation of this would require measurement of cell dimensions. If this is the case, it suggests that hypertrophy is confined to infected cells but that hyperplasia is the defining factor that distinguishes resistance.

There was no evidence for vertical resistance as defined by van der Planck in 1963 even in cultivars of cabbage that did not develop macroscopically visible galls. Gall formation, not infection of the root hairs, is the critical stage reduced in resistance and it is quantitative rather than qualitative and sensitive to pathogen populations, typical of horizontal resistance. Gall formation leads to the disruption of the cortex and vascular tissue, which in turn leads to reductions in transport of water, minerals and photosynthates in the plant and hence wilting, stunting and chorosis (Agrios, 2005; Karling, 1968).

Many genomic and proteomic studies on *P. brassicae* infection have focused on early stages of infection, generally while the pathogen invades the root hairs and disseminates through the cortex to form cells with plasmodia (e.g. Cao et al. 2008; Kong Kaw Wa 2009). Galls are formed by both hypertrophy and hyperplasia but hyperplasia seems to be the critical stage, as discussed previously. This stage is therefore an important target in control. Few until recently have investigated this critical stage using molecular tools (e.g. Malinowski et al. 2012; Siemens et al. 2006; Siemens et al. 2009a and b; Siemens et al. 2011; Wu et al. 2012).

The lack of gall formation in resistant cabbage plants even in the presence of much cell infection suggests that the regulation of cytokinin is a critical step and that it is less the increased proportion of cells infected and more the hyperplasia induced at plasmodial nucleus proliferation that result in galls swelling, well after initial infection. Several important steps in the molecular control of and by the pathogen occur at this stage and suggest that the plasmodia induce the vascular cambium and phloem parenchyma to divide, causing islands of cambial activity and breaking up the stele (Malinowski et al. 2012). Finding a way to prevent this is the key to devising lasting control of clubroot and simultaneous studies of morphology and molecular biology are essential.

Chapter 7. Overview

When research for this thesis commenced, it was known that Australia had a very wide diversity of pathotypes of *P. brassicae* (Donald et al. 2006a) but nothing was known of the genotypes. It was also known that the ECD types frequently differed from test to test but whether this was because of genetic variation or difficulties in the reliability of the ECD assay was not understood, because the genetic variation of Australian populations had not been investigated apart from limited work with microsatellites (Faggian 2002). The morphological consequences of variation in Australian pathotypes on the course of infection in host plant roots had been investigated previously, but only with *B. oleracea* (cauliflower) (Donald et al. 2008) and *A. thaliana* (Agarwal et al. 2011), but not with other host species.

From the research in the present study, it is now known that:

- new ECD pathotypes and ECD types different from previous testing have been found
- genotypes of Australian populations of *P. brassicae* are very diverse and do not cluster in the same way as ECD pathotypes
- genotypes of these Australian populations change with each generation of plant passage
- mixing genotypes in inocula results in new genotypes
- genotypes of some single-spore isolates vary from generation to generation and with accession
- the infection process differs with level of resistance in cabbage and Chinese cabbage, not at initial root-hair infection but later, during the colonisation of the root cortex
- resistance is associated with smaller proportions of infected cells and a predominance of infected cells containing plasmodia rather than resting spores.

As these findings have consequences for further research, they are discussed further below with recommendations for future research. Previous references have been omitted as they have been discussed extensively in relevant chapters.

7.1 ECD pathotype variation

The finding of one new pathotype in only these ten populations, nine of which had been tested previously, confirms the great diversity of the Australian populations of *P. brassicae*. While no testing system based on pathogen-host will ever produce identical results, the variation seems real rather than an artefact, and raises the question of why Australian

populations are so variable. Australian soils are nutrient-poor on a world scale and such soils tend to produce high biodiversity, not only on the plants that grow there but also in the microbes in the soil and the animals associated with them. This, however, does not explain the lack of stability from one ECD test to the next for some populations and it is likely to be the result of underlying genotype variation (Lambers et al. 2013).

Future research should further explore the diversity of the Australian populations in more depth, in particular to compare how much they change over time in the field and in frozen storage to see where the changes occur and with what frequency. Testing at annual intervals would show if pathogenicity is lost and if the loss is greater in frozen storage than in the field. This could improve the ability to conduct epidemiological research into changes in Australian populations with time and to store resting spores in a viable state. This would, however, be both time-consuming and space-demanding.

7.2 Genotype variation

Genotype variation in these ten Australian isolates appeared even greater than pathotype variation, partly because of the highly discriminating nature of both RAPD and microsatellite primers. As a reduced set of primers still gave satisfactory clustering, they should be used in future to type many more Australian populations and compare them with others worldwide to assess their relationships and trace their origins as in **Figure (7.1)**, which shows the high genotype diversity even between the Australian populations and the single-spore isolates used here. The high genotype diversity in *P. brassicae* indicates a state of rapid evolution of the pathogen in Australia.

Unlike many other parts of the world, in which clubroot has been found for many years. It is not known exactly when clubroot first occurred in Australia but the high diversity is not typical of introduced microbes and a form of it may have pre-dated European settlement in the 18th century. Genotyping may elucidate this, but only if genotypes are stable, as ECD pathotypes were not.

The lack of correspondence between genotype and pathotype clustering may be a consequence of the small number of populations tested. Ten populations may have been too few to allow for the emergence of a banding pattern linking particular pathotypes to genotypes. It would be desirable for genotyping to be conducted simultaneously with ECD pathotyping so that any individuals or clusters can be related. There is no *a priori* reason to

expect that they will be related, as genotyping methods typically depend on randomly repeated non-coding motifs in the genome whereas pathotyping depends on coding regions of both pathogen and host, but particular amplicons have been genetic markers for aggressiveness in fungi such as *Ophiostoma ulmi*, the cause of Dutch elm disease (Jeng et al. 1991; Et-Touil et al. 1999), and larger-scale testing may reveal similar markers, internationally if not locally.

Further research should investigate if any of this high genotype diversity has its origin in host plant DNA, as that would negate these results and conclusions, by using other plant-specific primers in PCR to see if amplicons are formed in DNA extracted from resting spores from galls. An alternative would be to infect transformed *Brassica* species capable of transient expression of a stable marker, e.g. *gus* (Christey and Sinclair 1992), and examine if it is expressed in resting spores or subsequent zoospores and plasmodia.

7.3 Generational change

The value of genotyping populations is largely lost if the genotypes are not stable, both from generation to generation and in mixed inocula, as was the case with the few populations of contrasting genotypes and pathotypes tested. The rapid changes in genetic diversity are similar to that seen in *O. ulmi* (Brasier 1988), and indicates a pathogen in a process of constant evolution. The extensive changes in genotype profile are probably due to multiple occurrences of genetic recombination in the *P. brassicae* life cycle every time an infection produces resting spores (Ingram and Tommerup 1972). Complete renewal of genetic profile with every infection makes genotyping expensive and perhaps worthless for use in attempts to breed resistance, as the target population constantly changes and markers for pathogenicity are likely to change with each generation. Breeding for resistance therefore needs to target invariant common genes rather than those that are pathotype- or genotype-specific, as is the case in currently known resistance to *P. brassicae*.

As only a few populations were tested here, similar tests should be carried out with a wider range of populations and through more generations to confirm these results and to test their limits of variation. These should be combined with pathotype testing, for reasons explained previously. It would also be desirable to produce some single-spore isolates from a population with a distinctive profile, e.g. population 3, to see if the profiles seen are the sum of those of their single-spore isolates.

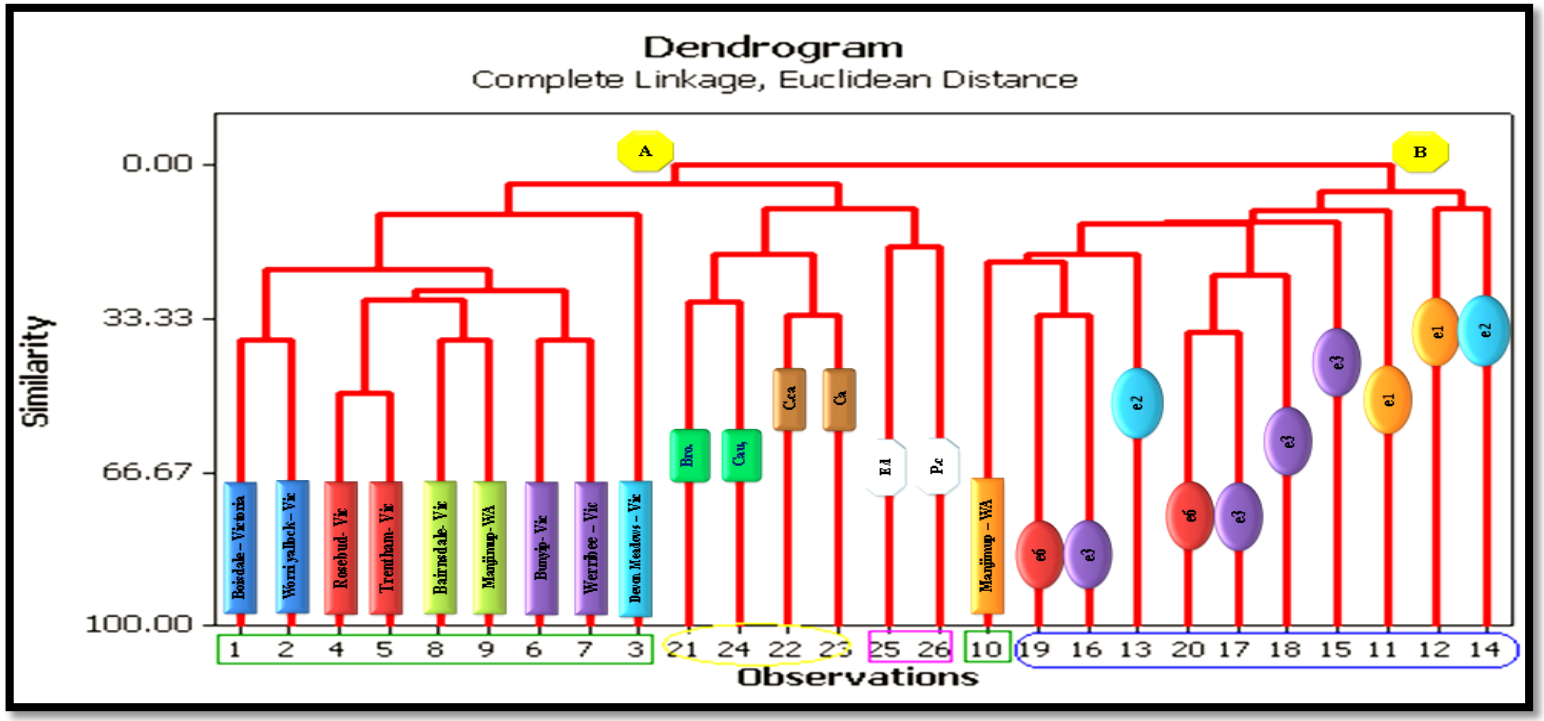

Figure 7.1: Cluster analysis of 26 different samples from RAPD and microsatellite profiles using complete-linkage and squared Euclidean distance, generated in Minitab 16. The numbers from left to right are as follows:1.Population No.1 (Boisdale – Victoria), 2. Population No.2 (Worriyallock – Vic), 3. Population No.3 (Devon Meadows – Vic),4. Population No.4 (Rosebud- Vic), 5.Population No.5 (Trentham- Vic), 6.Population No.6 (Cora Lynn Bunyip- Vic), 7.Population No.7 (Werribee - Vic), 8. Population No.8(Lindenow- Bairnsdale – Vic), 9. Population No.9 (Manjimup - Western Australia), 10 Population No.10 (Manjimup – WA), e1a old single spore isolate, e1b new single spore isolate, e2a old single spore isolate, e2b new single spore isolate, e3a old single spore isolate, e3b old single spore isolate generated in Victoria, Australia, e3c new single spore isolate regenerated from number16 in Victoria, Australia, e3d new single spore isolate imported from Germany, e6a old single spore isolate , e6b new single spore isolate, broccoli, Chinese cabbage, cabbage and cauliflower, *Exophiala dermatitidis* and *Penicillium chrysogenum*.

7.4 Single-spore isolates

The generational changes observed with single-spore isolate e3 were significant, unexpected and largely negate the value of using this isolate in challenge experiments to achieve constancy and find genes useful in plant breeding. The changes in genotype between accessions with all four single-spore isolates of the ‘e’ series were also unexpected and causes for caution. The explanation for both is probably the bulking-up process of plant passage and frozen storage, the first with its obligatory genetic recombination and the second with its potential problems of accelerated mutation during the freeze-thaw process.

Future research should genotype as many accessions as possible of the ‘e’ series through space and time to estimate how much change has taken place from the first galls. Further experiments through generations of plants and with mixed isolates, which this research could not perform due to the AQIS permit conditions, are needed to confirm the changes observed with these isolates. The discovery of genotype diversity in the single-spore isolates used in this research does not imply that all single-spore isolates show the same genotype diversity, but it would be interesting to test them using the selected primer set used here. The results will determine if single-spore isolates have value that exceeds the difficulties of producing them.

7.5 Infection process

Events observed in light microscopy in cabbage and Chinese cabbage conformed to those observed by others in a variety of crucifers. Root-hair stages of infection by primary zoospores was uniform and invariant but differences in virulence of the pathogen and resistance of the host caused differences in cortex and stele invasion, as seen previously in other species. High virulence in populations increased the proportion of cells infected in the cortex and stele and any degree of resistance delayed gall development and reduced resting spore production, as found by others, largely due to reductions in hyperplasia.

Further immediate research should quantify the effects of pathogen and host on the proportion of cells infected in cabbage. It would be desirable to examine the materials prepared by transmission electron microscopy to see if ultrastructural features resemble those seen previously, in particular myxamoebae, amyloplasts, lipid droplets and nuclei in both pathogen and host. In particular, the older plasmodia in resistant plant cells show distinctive

structures that are only resolvable with greater resolution. The use of pathogen population x host cultivar combinations elucidated the effects of each and its use in future is highly desirable. Any future research should take samples simultaneously for histology and gene expression, as the combination of techniques is more powerful than each alone.

7.6 Conclusion

This research has produced some unexpected new and significant information on the high genotype diversity and low genotype stability of Australian populations and single-spore isolates of *P. brassicae*. It has also shown in two host species that plant resistance and virulence in the pathogen are expressed at the secondary phase of infection of host cortex and stele tissues rather than at the primary infection phase in root hairs. This thesis has therefore advanced our knowledge and understanding of clubroot.

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Appendix Ch. 6.

Table A6.7a. The number of cells with resting spores and number of cells with plasmodium for cabbage (*Brassica oleracea*) with population No. 1 at 28 dai.

Reading No.	Susceptible host			Resistant host		
	Resting spores	Plasmodium	Intact cells	Resting spores	Plasmodium	Intact cells
1	19	1	13	0	23	31
2	21	2	6	1	28	23
3	26	1	19	0	20	17
4	29	2	8	0	26	20
5	30	1	7	1	21	13
6	27	3	12	2	18	18
7	21	1	10	0	19	22
8	30	3	6	0	30	16
9	19	0	14	0	27	11
10	18	0	10	0	22	12
11	19	0	9	1	28	34
12	26	2	11	0	23	20
13	29	2	19	0	40	16
14	18	0	12	0	22	13
15	22	3	20	0	30	19
16	26	1	17	1	23	20
17	29	0	15	1	26	33
18	24	2	10	0	28	20
19	19	0	16	0	11	27
20	21	1	10	0	19	28
21	31	0	18	1	28	11
22	22	3	16	0	29	5
23	28	0	19	0	26	10
24	29	0	10	0	22	2
25	21	2	27	1	27	4
26	18	0	15	0	28	6
27	20	4	19	1	30	19
28	24	2	18	2	19	11
29	26	1	22	0	21	17
30	28	3	8	0	23	20
31	29	2	16	0	27	17
32	31	3	10	2	30	16
33	24	4	18	1	22	13
34	31	1	15	0	28	10
35	19	1	22	1	24	14
36	26	0	29	0	27	11
37	28	2	11	2	30	9
38	18	2	10	0	21	13
39	17	1	19	0	20	15
40	28	2	18	0	26	17
41	30	3	10	2	28	10
42	21	2	26	0	21	13
43	24	2	10	0	28	11
44	27	1	11	1	20	22
45	25	3	13	1	19	14
46	22	1	11	4	30	3
47	21	0	16	0	28	7
48	26	1	8	0	21	11
49	20	2	18	1	26	8
50	32	1	7	0	21	10

Table A6.7b. The number of cells with resting spores and number of cells with plasmodium for cabbage (*Brassica oleracea*) with population No. 1 at 28 dai.

Reading No.	Susceptible host			Resistant host		
	Resting spores	Plasmodium	Intact cells	Resting spores	Plasmodium	Intact cells
51	33	1	13	1	21	14
52	28	1	32	0	26	19
53	25	1	19	0	22	20
54	28	0	22	0	31	11
55	19	0	28	0	25	23
56	31	0	11	2	30	18
57	26	2	15	0	32	20
58	23	1	19	0	25	12
59	28	0	22	0	31	21
60	26	2	31	1	29	16
61	30	0	20	1	33	19
62	21	1	14	1	26	11
63	26	2	11	1	28	17
64	30	1	18	0	20	19
65	20	0	16	0	24	20
66	31	3	14	0	24	17
67	28	0	17	0	28	22
68	22	0	11	0	19	18
69	25	0	10	0	33	20
70	30	0	9	1	24	25
71	22	0	11	2	29	22
72	19	0	20	1	26	19
73	21	2	18	1	22	20
74	30	1	9	0	26	29
75	29	1	17	0	32	20
76	27	1	14	0	30	18
77	21	0	10	0	24	20
78	29	0	12	0	30	17
79	20	0	19	1	30	20
80	22	0	16	2	28	23
81	28	0	11	3	30	18
82	25	2	20	1	24	20
83	22	0	15	1	19	23
84	29	2	19	2	22	20
85	20	0	12	0	26	12
86	26	0	17	0	29	20
87	28	0	11	0	24	29
88	33	0	9	4	28	12
89	29	0	16	0	21	17
90	19	1	11	0	29	0
91	27	1	20	1	27	18
92	30	0	11	0	19	27
93	28	0	20	3	31	12
94	25	0	23	1	11	20
95	28	0	18	1	19	16
96	30	1	9	2	31	19
9	31	2	11	1	24	20
98	29	1	6	0	27	16
99	26	0	19	0	21	18
100	30	0	11	1	26	23

Table A6.7c. The number of cells with resting spores and number of cells with plasmodium for cabbage (*Brassica rapa*) with population No. 1 at 28 dai.

Reading No.	Susceptible host			Resistant host		
	Resting spores	Plasmodium	Intact cells	Resting spores	Plasmodium	Intact cells
101	26	2	21	0	30	20
102	30	1	10	1	28	23
103	26	0	16	1	31	22
104	20	0	24	1	22	17
105	28	0	12	1	29	13
106	29	1	10	1	22	19
107	27	1	18	0	20	30
108	19	0	29	0	28	28
109	25	1	17	0	20	18
110	22	0	29	2	31	27
111	30	0	12	1	26	16
112	29	0	12	0	28	23
113	27	0	19	0	31	18
114	20	2	16	0	19	19
115	27	1	20	0	24	20
116	22	0	17	3	29	12
117	26	0	16	0	28	18
118	31	0	16	0	30	20
119	23	0	10	0	26	26
120	27	0	22	1	29	21
121	31	1	19	1	31	23
122	28	1	16	1	28	20
123	30	1	20	0	27	19
124	29	1	19	0	22	25
125	26	1	17	0	31	18
126	28	1	20	0	28	16
127	30	0	23	0	25	10
128	22	1	16	3	22	19
129	31	2	18	2	26	22
130	27	0	20	0	29	26
131	29	2	24	0	20	19
132	27	0	11	0	29	16
133	21	0	19	0	25	15
134	28	1	16	1	29	20
135	30	2	11	0	31	2
136	24	1	19	1	22	5
137	21	0	20	0	34	11
138	29	3	23	1	20	23
139	51	0	17	0	29	19
140	23	1	19	0	25	20
141	27	0	20	0	23	23
142	19	0	23	0	23	19
143	30	4	19	0	31	10
144	25	2	16	2	29	15
145	29	0	22	3	25	13
146	30	0	18	0	29	20
147	19	1	22	0	19	16
148	28	1	20	0	23	19
149	22	1	19	0	30	14
150	28	1	10	0	28	26
Total	**3868**	**142**	**2406**	**89**	**3854**	**2625**
Proportion	**60.3%**	**2.2%**	**37.5%**	**1.4%**	**58.8%**	**40%**

TableA 6.8a. The number of cells with resting spores and number of cells with plasmodium for cabbage (*Brassica rapa*) with population No. 4 at 28 dai.

Reading NO.	Susceptible host			Resistant host		
	Resting spores	Plasmodium	Intact cells	Resting spores	Plasmodium	Intact cells
1	52	5	1	3	46	4
2	49	0	2	2	51	2
3	38	0	0	5	44	5
4	44	1	3	8	51	7
5	49	4	4	3	52	2
6	51	3	1	2	54	9
7	60	4	7	2	48	0
8	54	0	2	8	50	7
9	49	6	8	1	46	5
10	52	2	5	5	49	3
11	67	2	4	1	39	1
12	51	0	9	2	44	5
13	49	3	3	18	61	7
14	44	2	2	3	55	4
15	39	3	8	1	58	8
16	60	4	5	2	43	3
17	56	1	4	0	49	0
18	55	1	2	1	43	1
19	58	5	6	5	49	2
20	61	0	3	6	55	4
21	53	2	1	4	41	0
22	57	3	4	4	59	6
23	60	1	7	1	32	3
24	53	6	4	3	51	5
25	49	3	4	1	49	8
26	53	0	12	10	52	2
27	41	5	1	1	58	3
28	50	6	5	2	72	5
29	44	6	7	3	60	8
30	61	9	9	5	51	6
31	55	0	5	9	59	9
32	58	9	2	4	47	3
33	53	0	2	8	56	6
34	51	5	0	10	62	1
35	54	0	9	8	50	6
36	49	6	5	13	54	9
37	38	3	3	5	41	5
38	43	2	5	6	63	3
39	55	4	6	2	48	5
40	52	2	3	6	59	2
41	53	4	6	3	34	7
42	49	0	5	12	50	5
43	61	5	4	1	41	4
44	49	1	7	6	48	1
45	52	4	3	8	50	7
46	49	1	7	9	44	4
47	50	8	3	6	40	9
48	54	10	1	4	41	2
49	51	7	9	14	49	3
50	57	13	3	2	58	8

Table A6.8b. The number of cells with resting spores and number of cells with plasmodium for cabbage (*Brassica rapa*) with population No. 4 at 28 dai.

Reading No.	Susceptible hosts			Resistant host		
	Resting spores	Plasmodium	Intact cells	Resting spores	Plasmodium	Intact cells
51	59	13	3	6	49	4
52	63	5	0	2	50	2
53	49	2	1	0	44	1
54	51	2	0	8	62	7
55	60	0	6	3	59	4
56	53	0	3	0	47	6
57	49	0	1	5	49	8
58	52	8	4	4	46	4
59	70	4	2	0	59	2
60	48	6	0	3	46	4
61	61	2	2	2	51	7
62	51	6	1	5	46	4
63	47	1	4	8	52	2
64	60	1	1	0	42	7
65	41	1	2	8	49	5
66	43	5	7	1	39	2
67	49	3	4	2	42	7
68	47	6	0	3	45	6
6	44	4	3	12	60	5
70	52	4	5	5	44	7
71	40	3	7	0	51	7
72	39	3	4	2	49	5
73	51	9	2	8	51	4
74	52	8	5	1	47	3
75	49	3	1	20	60	2
76	51	0	8	5	52	4
77	49	2	2	0	35	6
78	47	2	3	0	51	7
79	44	2	1	9	58	3
80	47	0	5	0	48	1
81	41	1	4	2	43	5
82	50	0	1	2	44	8
83	49	0	6	3	58	5
84	51	6	1	2	50	8
85	48	4	6	0	43	6
86	39	1	4	11	55	4
87	41	3	8	0	49	3
88	46	3	4	1	40	5
89	44	0	3	1	54	2
90	56	9	1	3	48	5
91	44	11	8	1	44	7
92	52	8	2	13	51	1
93	50	4	1	1	34	7
94	49	0	4	1	43	10
95	51	8	0	9	49	4
96	49	6	3	10	47	6
97	46	0	3	8	53	9
98	42	32	2	4	48	4
99	60	12	0	9	51	7
100	47	2	6	3	44	9
101	44	5	4	1	39	3

Table A6.8c. The number of cells with resting spores and number of cells with plasmodium for cabbage (*Brassica rapa*) with population No. 4 at 28 dai.

Reading No.	Susceptible hosts			Resistant host		
	Resting spores	Plasmodium	Intact cells	Resting spores	Plasmodium	Intact cells
102	56	4	1	3	45	6
103	47	9	8	3	51	9
104	53	2	5	2	48	6
105	50	15	3	0	40	5
106	43	2	6	12	62	8
107	49	6	9	8	59	6
108	60	11	2	6	55	3
109	54	2	3	8	57	9
110	49	0	0	0	49	2
111	47	0	4	0	40	0
112	58	4	1	1	55	5
113	43	2	4	4	52	2
114	60	7	1	1	46	7
115	55	5	7	2	58	4
116	39	0	0	5	63	8
117	51	1	2	0	41	0
118	59	1	0	2	56	6
119	53	1	2	4	50	4
120	55	5	1	6	58	8
121	49	0	6	0	48	2
122	44	3	5	2	57	10
123	58	5	8	4	53	4
124	42	0	0	0	55	7
125	48	0	2	3	59	0
126	54	6	4	0	48	7
127	63	16	1	11	60	4
128	49	0	0	9	53	6
129	49	3	5	1	48	3
130	64	6	3	14	57	5
131	50	5	7	9	55	8
132	53	8	2	3	48	6
133	49	3	0	9	51	5
134	53	5	1	7	54	4
135	51	0	2	1	47	6
136	55	1	3	4	49	4
137	49	1	1	11	58	7
138	60	11	1	9	58	9
139	57	7	4	3	49	8
140	55	5	7	0	46	5
141	59	8	3	14	59	3
142	50	6	0	4	48	2
143	69	18	5	8	57	5
144	65	10	2	10	51	6
145	58	2	1	9	63	4
146	70	16	7	10	62	0
147	48	0	6	0	49	7
148	56	1	0	7	50	3
149	54	4	1	2	40	2
150	63	7	0	8	59	0
Total	**7734**	**625**	**525**	**694**	**7752**	**718**
Proportion	**87.1%**	**7%**	**5.9%**	**7.7%**	**84.3%**	**8%**

Table A6.9a. No. of cells with resting spores, plasmodia and intact (uninfected for Chinese cabbage (*Brassica rapa*).

Reading No.	Susceptible host			Partially resistant host		
	Resting spores	Plasmodium	Intact cells	Resting spores	Plasmodium	Intact cells
1	9	4	11	3	15	9
2	12	3	6	0	28	5
3	37	0	9	0	19	6
4	27	1	4	1	24	7
5	26	0	8	0	21	10
6	22	0	6	0	19	14
7	16	1	4	0	18	34
8	15	3	8	0	12	24
9	14	0	12	1	22	16
10	27	5	8	0	28	15
11	17	2	6	0	14	5
12	22	0	4	0	12	2
13	30	0	10	0	16	9
14	22	0	6	0	26	16
15	2	26	4	1	22	11
16	22	9	7	0	19	9
17	18	1	4	2	18	12
18	36	18	11	1	28	7
19	34	2	20	0	22	8
20	41	1	7	0	21	5
21	33	1	13	0	13	9
22	22	0	4	0	11	6
23	7	0	8	0	14	4
24	23	0	10	0	16	11
25	15	0	18	0	20	17
26	21	0	9	0	13	6
27	26	14	6	0	9	9
28	47	2	11	0	13	11
29	16	1	7	0	16	4
30	12	0	3	0	11	16
31	23	1	6	0	9	19
32	42	3	8	0	18	10
33	33	1	9	0	21	16
34	26	0	7	0	29	11
35	50	0	5	0	13	9
36	12	0	21	2	27	13
37	41	6	17	1	25	7
38	39	3	4	1	30	5
39	27	0	8	1	19	3
40	37	0	3	1	10	4
41	23	0	8	0	27	11
42	30	0	6	0	38	13
43	22	2	10	0	22	9
44	28	1	16	0	19	15
45	31	19	10	0	26	14
46	29	0	8	0	15	12
47	43	0	12	2	33	4
48	27	0	9	2	26	3
49	20	3	6	1	20	11
50	31	0	8	0	19	16

Table A6.9b. No. of cells with resting spores, plasmodia and intact (uninfected for Chinese cabbage (*Brassica rapa)*.

Reading No.	Susceptible host			Partially resistant host		
	Resting spores	Plasmodium	Intact cells	Resting spores	Plasmodium	Intact cells
51	21	1	11	0	36	8
52	30	0	9	0	11	10
53	46	4	7	1	22	7
54	29	0	10	2	14	4
55	41	3	5	1	29	9
56	36	0	8	3	36	11
57	12	2	10	0	11	20
58	39	0	7	0	21	14
59	30	0	9	0	18	16
60	19	1	10	2	30	9
61	29	0	5	0	22	7
62	50	2	8	3	40	8
63	43	3	10	0	11	19
64	32	0	3	0	16	14
65	29	0	7	6	18	10
66	18	3	11	0	29	11
67	47	6	9	1	33	9
68	39	0	20	4	11	10
69	28	0	3	0	16	21
70	40	3	9	0	19	17
71	36	0	11	0	22	9
72	28	0	5	0	14	20
73	26	0	8	1	21	18
74	20	2	10	2	18	22
75	34	0	16	1	6	19
76	52	3	10	0	14	18
77	16	5	7	1	18	21
78	42	11	7	2	29	11
79	51	16	5	0	22	18
80	39	0	11	0	18	14
81	32	0	3	0	20	10
82	50	4	2	0	11	23
83	42	0	1	0	16	15
84	38	0	7	0	21	10
85	31	0	9	1	17	16
86	29	1	6	0	19	13
87	40	13	5	0	11	20
88	33	0	9	0	15	17
89	36	3	10	0	18	19
90	28	1	4	0	20	23
91	31	0	11	1	22	21
92	39	0	8	2	17	19
93	11	0	13	0	13	23
94	42	0	4	1	20	21
95	29	13	10	1	18	19
96	33	2	12	0	23	20
97	22	4	9	0	26	9
98	19	0	12	0	11	15
99	46	9	3	0	19	18
100	42	1	7	1	14	27

Table A6.9c. No. of cells with resting spores, plasmodia and intact (uninfected for Chinese cabbage (*Brassica rapa)*.

Reading No.	Susceptible host			Partially resistant host		
	Resting spores	Plasmodium	Intact cells	Resting spores	Plasmodium	Intact cells
101	36	2	1	0	11	15
102	21	0	7	0	32	9
103	41	6	2	3	19	20
104	30	3	11	1	22	16
105	21	0	12	5	38	13
106	40	6	9	0	13	19
107	11	0	17	2	19	7
108	26	2	10	0	24	19
109	39	4	12	0	27	15
110	51	2	0	3	34	7
111	33	0	11	0	29	15
112	28	6	20	2	36	20
113	21	1	17	0	14	17
114	17	2	13	0	26	21
115	33	1	9	2	31	15
116	41	5	4	0	11	13
117	28	19	8	0	28	23
118	37	12	11	2	19	16
119	40	9	2	0	31	17
120	29	2	7	0	17	20
121	31	8	0	4	33	4
122	40	11	2	0	14	21
123	22	3	5	0	21	18
124	29	6	10	1	11	20
125	31	4	1	0	9	17
126	12	0	13	2	23	2
127	19	0	9	0	12	19
128	36	0	5	0	28	17
129	25	0	20	0	22	26
130	19	0	16	0	40	11
131	37	2	11	0	31	17
132	27	1	18	0	19	23
133	29	3	9	0	37	18
134	31	4	16	0	40	9
135	28	4	2	0	29	27
136	19	10	6	2	31	21
137	40	13	2	1	19	24
138	33	18	7	2	42	9
139	29	0	10	0	18	19
140	60	9	0	1	33	20
141	31	11	17	0	25	23
142	19	0	20	0	16	18
143	28	0	14	0	21	10
144	34	0	21	0	31	2
145	27	2	17	1	22	14
146	16	0	29	0	9	31
147	28	4	2	0	27	19
148	31	0	8	1	35	13
149	49	0	16	0	14	26
150	33	0	9	1	46	5
Total	**4476**	**444**	**1329**	**93**	**2301**	**2104**
Proportion	**71.6 %**	**7.1%**	**21.2%**	**1.7%**	**59.3%**	**39%**